International Journal for Computational Biology (IJCB)
Vol.7, No.1, Apr 2018, pp. 60~69
ISSN: 2278-8115

ATPase Domain of Heat Shock protein 70—isoform 2—(Hsp70-2) and their role in activating the adaptive immune response: An *in silico* approach

Gregorio Marchan
Centro de Medicina Experimental, Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela.

Article Info

Article history:

Received Feb 23rd, 2018
Revised Apr 10th, 2018
Accepted Apr 14th, 2018

Keyword:

Hsp70-2
ATPasa Domain
Breast cancer
Epitope
Fifth keyword
Adaptive immune response

ABSTRACT

A lot of molecules play fundamental roles in neoplasic processes and cancer. Heat shock proteins may enhance these pathologies and favor the protumoral milieu. However, a look at the literature tells us that these molecules intervene in both to promote or attack cancer cells. In the case of breast cancer is known that Hsp70 (isoform 2) improve its establishment and progression in the patient, and is possible that the ATPase domain of Hsp70-2 favors this disease. Thus, is relevant to know if this molecular region has immunogenic activity as well as which epitopes are essential to stimulate immune cells, and whether could induce the attack of the tumor mass. In this aim, the immunogenicity of ATPase domain of Hsp70-2 was studied *in silico*. The results suggest that the majority of the molecule had immunogenic epitopes that boosts the immune response through activation of B cells and T cells. However, *in vitro* synthesis and *in vivo* experimental studies to evaluate the efficacy of this therapeutic candidate are required to ensure safety in people.

Corresponding Author:

Centro de Medicina Experimental. Instituto Venezolano de Investigaciones Científicas (IVIC). Apartado 20632, Caracas 1020-A, Venezuela. Tel.: +58 (0) 2125041158. Email: gregoriomarchan91@gmail.com

How to Cite:

Marchan G. ATPase Domain of Heat Shock protein 70—isoform 2—(Hsp70-2) and their role in activating the adaptive immune response: An in silico approach. IJCB. 2018; Volume 7 (Issue 1): Page 60-69.

1. INTRODUCTION

Breast cancer (BC) is one of the leading causes of death of women worldwide and is listed as a serious problem in public health [1-2]. Breast cancers mostly may include two hystotypes: invasive type such as infiltrating ductal carcinoma (IDC) and non-invasive type, as Ductal Carcinoma in-situ (DCIS) [3]. The first one cause inflammatory breast cancer that is characterized by its fast and aggressive behavior and the patients have a 43% increased of risk of death. However, despite clinical differences, these cancers shared certain similarities and plasma membrane proteins are one them [4]. Therefore, these molecules serve as targets in 70% of cancer therapies at clinical and experimental level [5]. Thus, identification and study of new molecules associated to BC can aid to early diagnosis and enhance new therapeutics.

Heat shock proteins (Hsps) are molecular chaperones that may modifying the structures and interactions of other proteins [6]. They shift the balance from denatured, aggregated protein conformation toward ordered, functional conformation, and are particularly in demand when proteins are disordered by heat shock, oxidative stress, or other protein-damaging events [7-9], intervening both co-translationally and post-translationally, at the same time as improve cell life to such stress factors [10].

Few works focus in the association between Hsps and BC. But recently studies show that HSP27, HSP90 Y HSP70 can contribute in the establishing and progression of this pathology [11-15]. Regarding the HSP70 family, eight members have been identified with a high structural and functional homology but with

different intracellular localizations [16]. Nevertheless, during cell stress Hsps go to extracellular milieu, existing as free proteins or anchor in the plasma membrane in the context of cholesterol-rich microdomains (lipid rafts) [17-18].

Gabriele Multhoff et al. in 1995 made the first report of the selective expression of a form of Hsp70 on the plasma membrane of solid tumor cells—but not normal tissue—since then, we know that Hsp70 family is highly expressed on metastatic disease and is associated with both poor prognosis and low survival [19-21]. This is an important reason why membrane Hsp70 expression is consider as a universal, selective tumor-specific marker of aggressive disease [7]. In cancer the ATPase domain of Hsp70 is localized outside the plasma membrane [22] but until now how does it works at molecular level during disease is not well understood. A most recent research found that Hsp70 isoform 2 is over expressed in BC patients and involved in malignant properties, by which it may be potential candidate molecule for development of better BC treatment [23].

Due to the above, this paper evaluates the immunogenic capacity of the ATPase domain of Hsp70-2 as a possible inductor of the immune response promoted by B lymphocytes and T lymphocytes, using for this the *in silico* analysis given that have been demonstrated that this approach is one of the best tools for the design and evaluation of vaccines before starting the experimental study.

2. RESEARCH METHOD

2.1. Protein sequence retrevial

The protein sequence of Hsp70-2 was retrieved in FASTA format from Uniprot Knowledgebase data, have also used accession Nos. P54652. The ATPase domain (also known as the nucleotide binding domain (NBD) was identified in the same database (http://www.uniprot.org/blast/?about=P54652[2-389]&key=Region).

2.2. Optimization of the ATPase domain

Through Jcat server the NBD was optimized (http://www.jcat.de/Start.jsp), this software allows the generation of a codon adaptation by avoiding cloning sites for restriction enzymes and Rho-dependent transcription terminators [24]. Through the Vaxijen server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) I predict the immunogenicity. This is the first server for the independent prediction of antigen alignment, that is, it allows classifying the antigen essentially based on the physicochemical properties of the protein without resorting to the sequence of alignment [25].

2.3. mRNA improved

The nucleotide sequence of the protein was obtained from the Jcat server, and then it was introduced in the webserver Secuence Massager (http://www.attotron.com/cybertory/analysis/seqMassager.htm) to get the RNA sequence and finally the structure. The gene was analyzed by the RNAfold program (http://rna.tbi.univie.ac.at//cgi-bin/RNAWebSuite/RNAfold.cgi).

2.4. Secondary and tertiary structure prediction analysis

These analyzes were carried out with the "The Predict Protein server" (https://www.predictprotein.org), which predicts secondary structure elements and solvent accessibility using evolutionary information from multiple sequence alignments and a multi-level system [26]. The 3D structure of the ATPase domain of HSPA2 was executed on the I-TASSER online server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/), this is a hierarchical approach to protein structure and function prediction. It first identifies structural templates from the PDB by multiple threading approach LOMETS, with full-length atomic models constructed by iterative template fragment assembly simulations [27].

2.5. The physico-chemical parameters evaluation

The physico-chemical parameters such as theoretical isoelectric point (pI), molecular weight, total number of positive and negative residues, extinction coefficient, half-life, instability index, aliphatic index, and grand average hydropathy (GRAVY) were computed using the Expasy's ProtParam (http://us.expasy.org/tools/protparam.html).

2.6. Prediction of possible transmembrane sequences

Due to the fact that during BC the Hsp70-2 is overexpressed in the plasma membrane, it was necessary to determine if this molecule possesses a specific sequence that facilitates its anchor in this cellular region, for which the complete aminoacidic sequence was upload in the Octopus online server (http://octopus.cbr.su.se/index.php), which uses a novel combination of hidden Markov models and artificial

neural networks, and predicts the correct topology for 94% of the a dataset of 124 sequences with known structures [28].

2.7. Prediction of epitopes for B lymphocytes and T lymphocytes.

The amino acid sequence of interest was submitted to online servers to predict continuous and discontinuous epitopes for B lymphocytes, using BCPRED (http://ailab.ist.psu.edu/bcpred/) and Discotope (http://tools.iedb.org/discotope) respectively. The access code for Discotope was obtained through Protein Data Bank: 3i33 (HSP70-2: ATPase domain).

The prediction of epitopes for T cells was performed through CTLpred (http://www.imtech.res.in/raghava/ctlpred/).

2.8. Prediction of MHC binding peptides

The prediction of binding peptides to MHC class-I was carried out by applying Propred-I (http://www.imtech.res.in/raghava/propred1/), while the prediction of MHC class-II binding peptides was performed through the online server RANKEP (http://imed.med.ucm.es/Tools/rankpep.html).

2.9. Prediction of IgE epitopes and allergic sites

Additionally, using Algpred (www.imtech.res.in/raghava/algpred/) the sites with allergenic potential were predicted.

3. RESULTS AND ANALYSIS

3.1. Optimization of the ATPase domain

To date there have been no studies that indicate that the NBD to be effective in immunization processes. But its over-expression in several BC hystotypes becomes it in an ideal target for the development of immunotherapy. One of the most important steps in designing synthetic genes is codon optimization. Codon bias and CG content was calculated. The codon adaptation index (CAI) was 0.96 and the CG content was reduced from 66.40% to 40.89%, favoring in this way the increase of the mRNA stability of the molecule (Figure 1). The antigenic index was 5.2 that allow classifying this molecule as a possible tumor antigen.

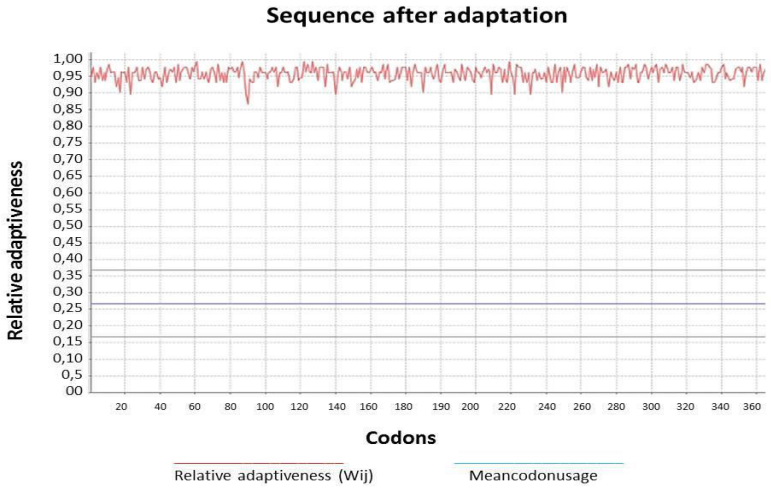

Figure 1. Graphical view of codon usage in optimized ATPase domain of Hsp70-2.

3.2. mRNA improved

Expression of proteins is significantly dependent on mRNA secondary structure, which confers beneficial roles such as regulation of gene expression. To perform this analysis, it was essential to use the minimum free energy for secondary structures formed by DNA molecules. The best structure released by RNAfold had a ΔG = -481.32 kcal/mol without formation of loops at the 5'-end.

In addition, this bioinformatics tool allowed visualizing the differences between the crude secondary structure of the RNA of the ATPase domain with respect to the secondary structure improved and thrown by the server using the minimum of free energy (MFE) necessary to reach an adequate folding (Figure 2). This Free energy minimizations can elucidates RNA secondary structure because it may aid in the determination of a

comparative sequence analysis model or suggest possible structures to test by site-directed mutagenesis or other methods [29].

The results from mRNA prediction indicated that the mRNA had enough stability for effective translation in the host. Therefore, based on this I can expect to increase the translation rates, half-lives, and transcript number of this molecule in the host.

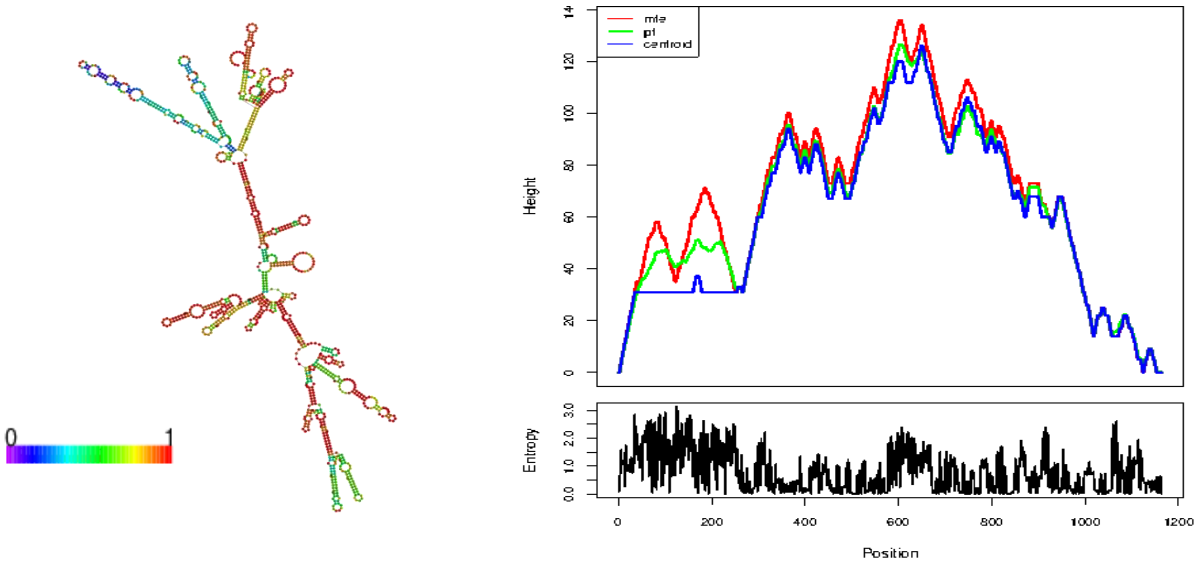

Figure 2. Prediction of ATPase domain RNA secondary structure by RNAfold, the color scale indicates the probability of base pairing formation (A). Representation of the MFE structure, the thermodynamic ensemble of RNA structures, and the centroid structure. Additionally is present the positional entropy for each position (B).

3.3. Secondary and tertiary structure prediction analysis

Three states of secondary structure were predicted: helix (H; includes alpha-, pi- and 3_10-helix), (beta-)strand (E = extended strand in beta-sheet conformation of at least two residues length) and loop (L) (Figure 3). Secondary structure is predicted by a system of neural networks with an expected average accuracy of more than 72% [26]. On the other hand, for each target, I-TASSER simulations generate a large ensemble of structural conformations, called decoys. To select the final models, I-TASSER uses the SPICKER program to cluster all the decoys based on the pair-wise structure similarity, and reports up to five models which corresponds to the five largest structure clusters. The confidence of each model is quantitatively measured by C-score that is calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. C-score is typically in the range of (-5, 2), where a C-score of a higher value signifies a model with a higher confidence and vice-versa [27], this was the case of the ATPase domain evaluated with a C-score of 1.50. Also, in these results I-TASSER simulations converge had less than 5 clusters generated indicating that the model has a good quality because of the converged simulations.

3.4. The physico-chemical parameters evaluation

The calculated molecular weight and the theoretical pI of the ATPase domain of HSPA2 was 42.4 KD and 6.63 respectively. The extinction coefficient of the molecule at 280 nm measured in water was 19. 035 M/cm. Half-life was estimated to be 1.9 h in mammalian reticulocytes (*in vitro*), >20 h in yeast (*in vivo*) and >10 h in *Escherichia coli* (*in vivo*). Similarly, the aliphatic index and Grand average of hydropathicity (GRAVY) were obtained: 83.99% and -0.334 respectively. The instability index classified the molecule as stable (instability index: 35.17).

3.5. Prediction of possible transmembrane sequences

The evaluation of this parameter did not yield results that indicate the presence of a transmembrane region in the natural conformation, that is, under normal physiological conditions (Figure 5). However, it is

presumed that during the beginning and evolution of BC could be induced post-translational modifications, and thus alter the protein conformation by incorporating new elements in their structure enhancing their anchoring to the plasma membrane [10, 22]. Besides, vesicular transport and ubiquitination-transport have been proposed as mechanisms [30-31], and they also serve for releasing of exosomes into the extracellular space.

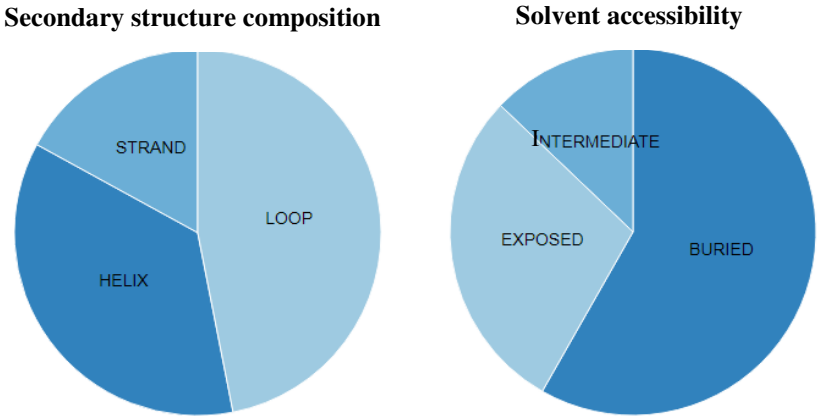

Figure 3. ATPase domain of Hsp70-2: Secondary structure composition an solvent accessibility.

Table 1. States of secondary structure predicted for ATPase domain of Hsp70-2: helix (H; includes alpha-, pi- and 3_10-helix) and (beta-)strand (E = extended strand in beta-sheet conformation of at least two residues length)

.

State	% H	%E
All-alpha	>45	<5
All-beta	<5	>45
Alpha-beta	>30	>20

Figure 4. Predicted 3D structure of ATPase domain of Hsp70-2 using I-TASSER software.

Various hematopoietic and non-hematopoietic cells secretes exosomes, including mostly macrophages, B and T lymphocytes, mast cells, platelets, alveolar lung cells, tumor cells and intestinal epithelial cells respectively. The tumor cells secreted exosomes rich in Hsp70, but the role of exosomal-associated Hsps

in cancer remain controversial because they can promote pro-inflamatory and anti-inflamatory effects depending on cellular origin and type of cancer [31-35], for example in BC the Hsp70-2 may aid progress the disease [23].

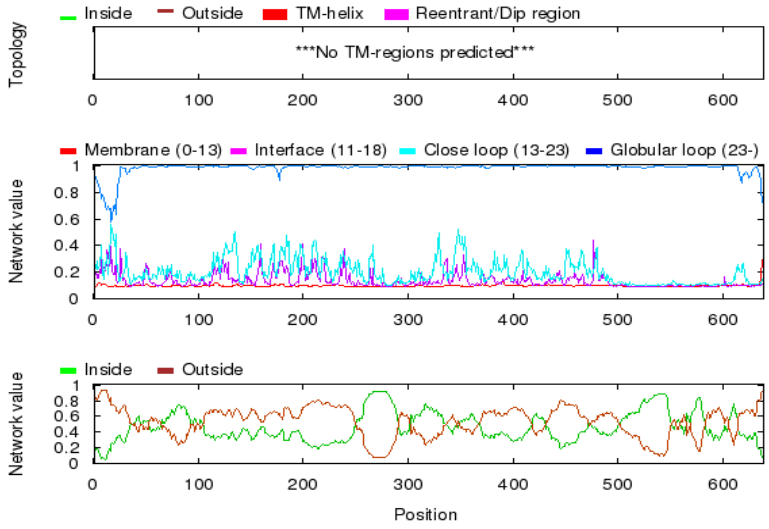

Figure 5. ATPasa domain of Hsp70-2: improving topology prediction by two-track ANN-based preference scores and an extended topological grammar using Octopus server.

3.6. Prediction of epitopes for T lymphocytes and B lymphocytes.

The prediction of CTL epitopes is represented in Table 2. The sequential epitopes were analyzed through the online Bcpred server. All 16-mers with Bcepred cutoff score >0.9 in B-cell epitopes were selected (Table 3). In addition, the conformational epitopes were chosen based on the following criteria: hydrophilicity, antigenicity, flexibility, accessibility, polarity, and exposed Surface (Table 4). The prediction of conformational epitopes (68), performed with Discotope is shown in Table 5. The identified epitopes on protein surface could interact easily with antibodies, and they were generally flexible.

Table 2. Prediction of T-cell epitope by CTLPred for ATPase domain of Hsp70-2.

Peptide rank	Start position	sequence	Score
1	25	KVEIIANDQ	1.000
2	108	KGETKTFFP	1.000
3	160	DAGTITGLN	1.000

Table 3. B-Cell epitopes from full length proteins using Bcepred for ATPase domain of Hsp70-2.

Position	Epitope	Score	Rank
245	EEFKRKHKKDIGPNKRAVRR	1	1
2	ARGPAIGIDLGTTYSCVGVF	0.998	2
98	GGKPKVQVEYKGETKTFFPE	0.994	3
357	NGKELNKSINPDEAVAYGAA	0.994	4
149	YFNDSQRQATKDAGTITGLN	0.932	5
28	IIANDQGNRTTPSYVAFTDT	0.862	6
74	IGRKFEDATVQSDMKHWPFR	0.832	7
222	KSTAGDTHLGGEDFDNRMVS	0.810	8
325	DAKLDKGQIQEIVLVGGSTR	0.744	9

Table 4. Epitopes predicted in HSPA2-ATPasa domain by different parameters.

Prediction	Sequence

Parametre	
Flexibility	IIANDQGNRTT, FRVVSEGGK, VEYKGET, AYFNDSQ, FEVKSTA, HLAEEFKRKHK, TACERAKRTLSSSTQ, IVLVGGS, FNGKELN, ILIGDKSE
Accessibility	ANDQGNRTTPSYVAFTDTERL, GDAAKNQ, KRLIGRKFEDATVQSDMKHWP, SEGGKPKVQVEYKGETKTFFPEE, TKMKEIAE, YFNDSQRQATKDA, AYGLDKKG, GEDFDNRM, LAEEFKRKHKKDIGPNKRAVRRLRTACERAKRTLSSS, TSITRARFEEL, TLEPVEKALRDAKLDKGQIQE, GSTRIPKIQK, NGKELNKSINPDE
Exposed Surface	KRLIGRK, KPKVQVEYKGETKT, SQRQATKD, AEEFKRKHKKDIGPNKRAVRR, ERAKRTL, RDAKLDK, RIPKIQK, NGKELNK, DKSE
Antigenic Propensity	GTTYSCVGVFQHGKV, HWPFRVVSE, PKVQVEYK, LNVLRII, NVLIFDLG, TFDVSILTIE,QIQEIVLVGGST
Hydrophilicity	ANDQGNRTTPS, GDAAKNQ, EDATVQSD, SEGGKPK, EYKGETKT, NDSQRQATKDAGT, DKKGCAGGEKN, VKSTAGDTH, GGEDFDNR, KRKHKKD, SSSTQAS, DKGQIQE, GDKSE
Polarity	FQHGKVEI, AKRLIGRKFEDAT, MKHWPFRV, KPKVQVEYKGETKTFFPEE, VLTKMKEIAE, GEDFDNRMVSHLAEEFKRKHKKDIGPNKRAVRRLRTACERAKRTLS, ITRARFEELNA, TLEPVEKALRDAKLDKG, RIPKIQK, NGKELNKSI, DKSE
Turns	YFNDSQR

3.7. Prediction of MHC binding peptides

Predicted peptides for binding to MHC-I over multiple alleles including MHC-2Kb, MHC-Db, MHC-2Dd, MHC-2Kd, MHC-2Kk, and MHC-2Ld were selected and showed in Table 6. RANKPEP online server was applied to predict binding peptides to class II MHC molecules as shown in Table 7.

3.8. Prediction of IgE epitopes and allergic sites

The protein sequence did not contain experimentally proven IgE epitope.

Table 5. Conformational B-cell epitopes from full length protein using Discotope server for ATPase domain of Hsp70-2.

Epitope	Position
HYS, GLY, THR, HIS, GLY, PRO, GLY, GLY, GLY, THR	24, 25, 48, 90, 99, 258, 293, 35, 110, 112
GLU, GLY, ASP	292, 332, 47
ALA, GLU, ASP	82, 111, 153
LYS, LYS, ASN	251, 253, 259
SER	365
GLN, SER, LYS	34, 86, 89
ARG	250
ASP, ASN, GLY	330, 358, 359
GLY, ASN	100, 152
LYS, ASP, ASP, ASN, GLN, MET, GLU, ASP, THR, GLU, LYS, LYS, LYS, PHE, SER	328, 355, 33, 36, 59, 62, 80, 81, 83, 98, 109, 113, 139, 151, 154
LYS, HIS, GLY, LYS	249, 252, 257, 260,
LYS	351
GLN, ASP, TRP, PRO	85, 87, 9, 92
ARG, VAL, SER	94, 96, 97
LYS	254
PRO	357
MET, PHE	88, 93

Table 5 continuation

GLN	157
GLU, ASP	247, 255
THR	65
GLU	246
ILE, ARG	256, 261
ALA	245

Table 6. The result of prediction for MHC-I epitopes.

MHC type	Start and End
MHC-Db	21-29, 71-74, 76-77, 80-83, 85-91, 133-140, 184-187, 189-197, 229-237, 273-280, 30-307, 357-361, 363-370, 372-379, 38-388, 395-401
MHC-Dd	2-8, 10-16, 18- 20, 22-24, 26-33, 37-38, 40-47, 133-139, 141-148, 156-162, 180-183, 185-191, 202-205, 207-208, 211-218, 224-230, 235-242, 250-257, 274-281, 310-316, 318-325, 339-346, 349-357, 359-360, 363-370, 376-382, 384-390, 392-399
MHC-Kb	22-27, 29-33, 37-38, 61-68, 92-99, 134-40, 164-171, 310-316, 318-324, 326-333, 363-370, 372-379, 387-394
MHC-Kd	39-46, 63-70, 78-82, 84-91,134-139, 141-148, 152-159, 180-181, 182-191, 201-208, 225-227, 229-236, 241-248, 310-317, 322-328, 330-337, 356-363, 391-392, 394-401
MHC-Kk	10-16, 20-27, 40-47, 62-64, 66-73, 88-93, 99-106, 124-131,136-139, 141-148, 150-153, 155-162, 175-181, 184-190, 193-200, 210-217, 222-223, 225-227, 228-232, 234-241, 251-252, 254-261, 288-293, 297-304, 310-317, 323-330, 356-363, 391-392, 394-401
MHC-Ld	2-4, 6-8, 10-17, 3-39, 55-62, 81-88, 102-110, 134-136, 138-145, 161-167, 194-201, 230-237, 241-244, 246-253, 258-266, 301-304, 306-313, 315-322, 366-373, 38-389

Table 7. The result of prediction for MHC-II epitopes.

Rank	Pos.	N	Sequence	C	Mw (Da)	Score	% Opt.
1	339	IVL	VGGSTRIPK	IQK	896.05	20.086	34.76 %
2	293	YEG	VDFYTSITR	ARF	1083.21	15.948	27.60 %
3	255	KKD	IGPNKRAVR	RLR	992.19	13.34	23.08 %
4	140	GKV	HSAVITVPA	YFN	876.02	12.798	22.14 %
5	166	TIT	GLNVLRIIN	EPT	993.21	11.413	19.75 %
6	333	KGQ	IQEIVLVGG	STR	909.09	10.523	18.21 %
7	244	SHL	AEEFKRKHK	KDI	1154.34	9.906	17.14 %
8	249	EFK	RKHKKDIGP	NKR	1060.26	9.556	16.53 %
9	377	GAA	VQAAILIGD	KSE	881.04	9.13	15.80 %
10	300	TSI	TRARFEELN	ADL	1117.24	8.92	15.43 %
11	310	LNA	DLFRGTLEP	VEK	1029.17	7.704	13.33 %
12	108	VEY	KGETKTFFP	EEI	1036.19	7.453	12.90 %
13	258	IGP	NKRAVRRLR	TAC	1150.4	6.476	11.21 %
14	68	NTI	FDAKRLIGR	KFE	1057.27	6.464	11.18 %
15	266	RRL	RTACERAKR	TLS	1072.26	6.411	11.09 %
16	169	GLN	VLRIINEPT	AAA	1036.24	6.298	10.90 %
17	354	LLQ	DFFNGKELN	KSI	1065.15	5.394	9.33 %
18	345	STR	IPKIQKLLQ	DFF	1062.36	4.788	8.28 %
19	163	DAG	TITGLNVLR	IIN	968.15	4.651	8.05 %

According to the in silico analyses presented above, the physico-chemical parameters evaluation indicates that this region of the Hsp70-2 protein is able to remain for a long period of time in bacterial cells (>10 h) which would be useful for its obtaining and purification in the laboratory. The presence of a high percentage of negatively charged amino acids (Glu and Asp) on the accessible surface and the high flexibility of the evaluated immunogenic epitopes suggests that these can interact with the basic pockets of the union groove of the major histocompatibility complex (MHC) molecules on leukocytes and thus activating immune cells, as B cells and T cells, against the ATPase domain of Hsp70-2. This could improve the attack to the tumor mass through antibodies production and activation of T helper and cytotoxic cells [36]. In this sense, antibodies can serve to promote antibody-dependent cytotoxicity (ADCC) and complement attack, while pro-inflammatory cytokines secretion of T helper cells may aid to establish an acute inflammatory state that favors the anti-tumoral effects [37]. However, are need *in vitro* and *in vivo* studies to check the efficacy and safety of this approach because it is known that in clinical trials a lot of vaccines deviate the "good" immune response to another worse, which has the ability to induce an unresponsiveness state that is promoted by antigenic presentation of the antigen by dendritic cells to T regulatory cells leading to suppress the immune response against cancer [37]. Additionally, the amount of loops and helix in the structure of this protein (Figure 4 and 5) reflects that it can have a wide versatility and, therefore, also variable functions, which will improve the tumor environment favoring its establishment and progression in the patient.

4. CONCLUSION

The several bioinformatics methods for immunogenicity prediction of the ATPase domain of Hsp70-2 protein demonstrated that epitopes of this region could induce B cell and T cell–mediated immune responses successfully. Therefore, is suggested as a potential candidate for modulation and reduction of tumor mass. As the next steps to complete this study on vaccine development, I recommend *in vitro* synthesis and *in vivo* experimental studies to evaluate the efficacy of this potential vaccine.

REFERENCES

[1] Siegel R, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin. 2016; 66:7–30.
[2] Howell A, Anderson A, Clark R, Duffy S, Evans D, Garcia-Closas M, et al. Risk determination and prevention of breast cancer. Breast Cancer Res. 2014; 16: 446.
[3] Makki J. Diversity of Breast Carcinoma: Histological Subtypes and Clinical Relevance. Clin Med Insights Pathol. 2015;8:23–31
[4] Suárez-Arroyo I, Feliz-Mosquea Y, Pérez-Laspiur J, Arju R, Giashuddin S, Maldonado-Martínez G, et al. The proteome signature of the inflammatory breast cancer plasma membrane identifies novel molecular markers of disease. Am J Cancer Res. 2016; 6(8): 1720–1740.
[5] Ziegler YS, Moresco JJ, Tu PG, Yates JR 3rd and Nardulli AM. Plasma Membrane Proteomics of Human Breast Cancer Cell Lines Identifies Potential Targets for Breast Cancer Diagnosis and Treatment. PLoS One 2014; 9: e102341.
[6] Freeman BC, Yamamoto KR. 2002. Disassembly of transcriptional regulatory complexes by molecular chaperones. *Science* 296: 2232–2235.
[7] Graham P, Henderson Brian, Extracellular cell stress (heat shock) proteins—immune responses and disease: an overview. Phil. Trans. R. Soc. B 373: 20160522.
[8] Hightower LE. 1991. Heat shock, stress proteins, chaperones and proteotoxicity. *Cell* 66: 191–197.
[9] Georgopolis C, Welch WJ. 1993. Role of the major heat shock proteins as molecular chaperones. *Ann Rev Cell Biol* 9: 601–634.
[10] Murphy E. The HSP70 family and cancer. Carcinogenesis vol.34 no.6 pp.1181–1188, 2013.
[11] Ciocca D, Calderwood S. Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. Cell Stress & Chaperones (2005) 10 (2), 86–103.
[12] Zorsi E, Bovini P. Inducible Hsp70 in the Regulation of Cancer Cell Survival: Analysis of Chaperone Induction, Expression and Activity. *Cancers* 2011, *3*, 3921-3956
[13] Sims J, McCready, Jay D. Extracellular Heat Shock Protein (Hsp)70 and Hsp90α Assist in Matrix Metalloproteinase-2 Activation and Breast Cancer Cell Migration and Invasion. PLoS One. 2011; 6(4): e18848.
[14] Cheng Q, Chang JT, Geradts J, Neckers LM, Haystead T, Spector NL, et al. Amplification and high-level expression of heat shock protein 90 marks aggressive phenotypes of human epidermal growth factor receptor 2 negative breast cancer. Breast Cancer Res. 2012;14:R62.
[15] Straume O, Shimamura T, Lampa MJ, Carretero J, Øyan AM, Jia D, et al. Suppression of heat shock protein 27 induces long-term dormancy in human breast cancer. Proc Natl Acad Sci U S A. 2012;109:8699–704.
[16] Rohde M, Daugaard M, Jensen MH, Helin K, Nylandsted J, Jäättelä M. Members of the heat-shock protein 70 family promote cancer cell growth by distinct mechanisms. Genes Dev. 2005;19:570–82.

[17] Bausero MA, Gastpar R, Multhoff G, Asea A. 2005 Alternative mechanism by which IFN-g enhances tumor recognition: active release of heat shock protein 72. J. Immunol. 175, 2900–2912.
[18] Broquet AH, Thomas G, Masliah J, Trugnan G, Bachelet M. 2003 Expression of the molecular chaperone Hsp70 in detergent-resistant microdomains correlates with its membrane delivery and release. J. Biol. Chem. 278, 21 601–21 606.
[19] Multhoff G, Botzler C, Wiesnet M, Muller E, Meier T, Wilmanns W 1995 A stress-inducible 72-kDa heatshock protein (HSP72) is expressed on the surface of human tumor cells, but not on normal cells. Int. J. Cancer 61, 272–279.
[20] Pfister K et al. 2007 Patient survival by Hsp70 membrane phenotype: association with different routes of metastasis. Cancer 110, 926–935.
[21] Gehrmann M et al. 2012 Immunotherapeutic targeting of membrane hsp70-expressing tumors using recombinant human granzyme B. PLoSONE 7, e41341.
[22] Gehrmann J, Marienhagen J, Eichholtz-Wirth H, Fritz E, Ellwart J. Dual function of membrane-bound heat shock protein 70 (Hsp70), Bag-4, and Hsp40: protection against radiation-induced effects and target structure for natural killer cells. Cell Death and Differentiation (2005) 12, 38–51.
[23] Jagadish N, Agarwal S, Gupta N, Fatima R, Devi S, Kumar V, et al. Heat shock protein 70-2 (HSP70-2) overexpression in breast cancer. Jagadish et al. Journal of Experimental & Clinical Cancer Research (2016) 35:150.
[24] Grote A, Hiller K, Scheer M, Munch R, Nortemann B, Hempel DC, et al. JCat: a novel tool to adapt codon usage of a target gene to its potential expression host. Nucleic Acids Res. 2005 Jul 1;33(Web Server issue):W526-31.
[25] Doytchinova I, Flower D. VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines. BMC Bioinformatics. 2007 8:4.
[26] Rost B, Yachdav G, Liu J. The PredictProtein server. Nucleic Acid Res. 32: 321-326 (2004).
[27] Zhang Y. I-TASSER server for protein 3D structure prediction. BMC Bioinformatics, 9: 40 (2008).
[28] Viklund H, Elofsson A. Improving topology prediction by two-track ANN-based preference scores and an extended topological. Bioinformatics. 2008.
[29] Mathews D, Disney M, Childs J, Schroeder S, Zuker M, Turner D. Incorporating chemical modification constraints into a dynamic programming algorithm for prediction of RNA secondary structure. Proc Natl Acad Sci U S A. 2004 May 11; 101(19): 7287–7292.
[30] Evdonin AL, Guzhova IV, Margulis BA, Medvedeva ND. 2004 Phospholipase C inhibitor, U73122, stimulates release of hsp-70 stress protein from A431 human carcinoma cells. Cancer Cell Int. 4, 2.
[31] Thery C, Zitvogel L, Amigorena S. 2002 Exosomes: composition, biogenesis and function. Nat. Rev. Immunol. 2, 569–579.
[32] Chaput N, Taıeb J, Schartz NE, Andre F, Angevin E, Zitvogel L. 2004 Exosome-based immunotherapy. Cancer Immunol. Immunother. 53, 234–239.
[33] Cordonnier M, Chanteloup G, Isambert N, Seigneuric R, Fumoleau P, Garrido C, Gobbo J. 2017 Exosomes in cancer theranostic: diamonds in the rough. Cell Adhes. Migr. 11, 151–163.
[34] van Eden W, van der Zee R, Prakken B. 2005 Heat shock proteins induce T-cell regulation of chronic inflammation. Nat. Immunol. 5, 318–330.
[35] Tanaka S et al. 1999 Activation of T cells recognizing an epitope of heat-shock protein 70 can protect against rat adjuvant arthritis. J. Immunol. 163, 5560–5565.
[36] Owen J, Punt J, Stranford J. Kuby: Immunology. 7th ed. New York: W. H. Freeman and Company 2013; pp. 835-31.
[37] Shevach E. Regulatory/suppressor T cells. In William P eds .Fundamental Immunology. 6th ed. Philadelphia, USA: Lippincott Williams & Wilkins 2008; pp. 1646-1315.