MiRNA-Regulated HspB8 as Potent Biomarkers in Low-Grade Gliomas

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Abstract
Low-grade gliomas LGGs are invasive brain tumors that occur mostly among young adults. Previous studies have shown that LGGs are characterized by IDH1/2 mutations; however, in some cases, cancer patients suffer from the low mutation rate of such genes. In this study, HspB8 is proposed as a new biomarker. On the basis of F-Census, HspB8 was correlated with gliomas; however, its role is yet to be determined. This study aims to identify the expression of HspB8 and its corresponding miRNA among LGG patients and assess its potential as a biomarker. The expression data are derived from The Cancer Genome Atlas (TCGA) project and are downloaded using TCGA Assembler. Then, HspB8–miRNA expression correlations and meta-analyses are conducted using MATLAB.

Results are validated via transcriptome analysis including miRNA-target side prediction and molecular docking simulation by RNAhybrid and PatchDock respectively. Results show the strongest negative correlation peaks at −0.417 (p value <0.05) found between HspB8 and mir-92a-1. Further transcriptomic validation also supports the interaction between the two RNA molecules denoted by negative free energy. However, their roles could not be validated due to the lack of research. Therefore, the results of this study might become a basis for further studies.

Keywords: Biomarker, HspB8, low-grade glioma, microRNA, transcriptomic.

Introduction

Low-grade gliomas LGGs diffusely infiltrate low and intermediate-grade gliomas including WHO grade II and III astrocytic tumors, oligodendrogliomas, and diffused gliomas (oligodendrogliomas)[7,28]. LGGs cannot be completely removed via surgery because of their invasiveness; the residual tumor may reoccur and become malignant causing the patients to experience tumor-related complications and even death[28,36].

According to Packer and Schiff[36], approximately 2,000 adults in the United States are diagnosed with LGGs each year and the male-to-female ratio is 1.58:1[11]. LGGs are particularly fatal for young adults and the average survival rate is 7 years[11]. Therefore, the pathogenesis of LGGs, particularly its biomarkers, must be studied. Biomarkers are measurable indicators of biological processes that objectively describe the normal and abnormal states of organisms[16,41].

In terms of diseases such as cancer, biomarkers differentiate the condition between cancer and healthy patients[19]. Biomarkers are disease specific (i.e. every biomarker is correlated with a certain disease); its identification has become an important aspect in developing personalized medicines[46]. Biomarkers have various types including gene and microRNA (miRNA) expressions[19]. Previous studies have shown that LGGs are denoted by mutations on isocitrate dehydrogenase (IDH) 1 and 2 genes[35]. Moreover, LGGs are acquired during early gliomagenesis followed by TP53 mutation or 1p/19q loss causing astrocytic or oligodendroglial phenotype respectively. However, the frequency of IDH1/2 mutations is yet to be defined because a low mutation rate of IDH1 has also been found among LGG patients[14].

These results show the limitations of proteomic-based studies particularly with the emergence of the transcriptomic-based ones[30]. As proteins mark the end of the central dogma, it undergoes many regulations. For example, more than 90,000 individual post-transcriptional modifications of proteins have been identified[22]. However, these regulations do not necessarily increase the inferred information[4]. In prokaryotes, the system has become saturated; the regulatory cost has become so high that it limits further genomic and functional regulations[30]. In this case, transcriptomic studies may become useful. RNA molecules also play an essential role in gene expression regulation[18]; therefore novel information which could not be obtained by proteomics, particularly in cancer pathology, can be unveiled.

In this study, we propose heat shock protein B8 (HspB8) as a new possible biomarker. HspB8 is a member of a human small heat shock protein (HSP) family that shares common features with HspBs such as stress inducibility and chaperone activity[8]. Previous studies[32,33] supported by the F-Census database[15] have shown that HspB8 is correlated with gliomas; however, its role as an oncogene or tumor suppressor gene (TSG) is yet to be determined. With the lack of research on HspB8 regulations in LGG, the transcriptomic approach might become useful. In this case, we correlate the expression of the gene and each type of human miRNA.

MiRNAs are small noncoding RNAs that generally downregulate its target gene by binding at the 3′ untranslated

region (UTR) of the gene[21]. If a strong negative correlation is obtained from the correlation analysis, then a direct association between HspB8 and its respective miRNA may exist in LGG; this information can be utilized in further research particularly in developing transcriptomic-based drugs. Our computational lab has developed a pipeline that combines correlational annotation and RNA structure elucidation studies to comprehend the mechanistic insight into miRNAs[1,37].

Moreover, the regulation of miRNA-regulated genes including the translation process and mRNA stability is also affected by argonaute (AGO) proteins[20]. AGO proteins are major constructors of RNA-induced silencing complex (RISC)[43]. AGOs comprise four domains: N-terminal, PIWI, PAZ, and MID; the latter two anchor the 3′ and 5′ ends of miRNAs respectively and guide it into SIRC. Then, the AGO-centered RISC binds to the 3′ UTR of the target mRNA, thereby inhibiting the translation process[24].

On the basis of this idea, assessing the binding feasibility between miRNAs, mRNAs, and AGO proteins is crucial. To perform such assessment, the molecular docking between the miRNA–mRNA duplex and the AGO protein becomes an option. By looking at the structures of the RNA and the protein, we can determine whether the interaction is likely to happen.

Based on The Cancer Genome Atlas (TCGA) dataset, this *in silico* study aims to identify the expression and feasibility of HspB8 with its corresponding microRNA (miRNA) among LGG patients, thus unveiling the potential of miRNA-regulated HspB8 as a biomarker in LGGs.

Material and Methods

A. Pre-study: The role of HspB8 (Entrez Gene ID: 26353) in LGGs was taken from the F-Census database[15] by searching the cancer genes on the basis of the cancer type (i.e. central nervous system).

B. Datasets: The dataset was derived from the TCGA project and stored in the GDC Data Portal[17]. In this analysis, the TCGA-LGG dataset was used.

C. Data Pre-processing: First, the metadata of the gene and miRNA expression datasets were downloaded from the GDC data portal. The keywords for retrieving the gene expression data are as follows: brain (primary site), TCGA-LGG (project ID), HTSeq–FPKM-UQ (workflow type), transcriptome profiling (data category), gene expression quantification (data type) and races (i.e. white, black or African–American and others). In this case, the keyword "others" comprises Asian, American–Indian or Native Alaskan and not reported.

The keywords for retrieving the miRNA expression data are as follows: brain (primary site), TCGA-LGG (project ID), transcriptome profiling (data category), miRNA expression quantification (data type), and races. The other parameters were left unchecked. As a result, six metadata files namely white-gene, black-gene, other-gene, white-miRNA, black-miRNA and other-miRNA were obtained. Then, the files were converted into CSV file format by using JSON to CSV converter[31] as a matter of preference.

Next, the metadata were imported into Python 3.6. As every patient is denoted by specific barcode (ID), the first 15 characters of all TCGA IDs from all metadata, which were patient-specific, were extracted and added into different lists. Then, the lists with the same race were matched: IDs that appear in both lists were retrieved. As a result, a list of IDs (patients) whose gene and miRNA expression data were available, was obtained.

These IDs were then input into TCGA Assembler 2.0.5[42,45], an R code implementation for downloading gene and miRNA expression data. In this case, only the first 12 characters of the ID were input, as required by the program. After the data were downloaded, they were imported into Microsoft Excel 2010 and saved in XLSX format.

D. Correlation Analysis: The gene and miRNA expression data from cancer patients were imported into MATLAB R2018a due to the unavailability of healthy patient data. Then, Spearman's correlation test was conducted between the HspB8 gene and every miRNA expression for each race. The HspB8–miRNA interactions with a Spearman's rho (R) and significance (p) value lower than -0.2 and 0.05 respectively were retrieved.

E. Meta-analysis: The significant gene-miRNA correlations that were found in all races were retrieved. Then, a meta-analysis was conducted on the basis of the fixed effects model[6] in MATLAB R018a. The strongest miRNA-regulated HspB8 was retrieved for validation.

F. Transcriptomic Validation: The sequences of the miRNA and 3′ UTR of the HspB8 mRNA were retrieved from the miRTarBase database[10]. The mature miRNA sequence was retrieved from the "Mature miRNA Information" column of one of the respective miRNA entries whereas the 3′ UTR of HspB8 was derived from the "Gene Information" column under the "Target Gene" tab of entries with HspB8 as the target gene.

Then, the miRNA-target site was predicted using RNAhybrid[38]. Afterward, the secondary and tertiary structures of the miRNA, gene, and miRNA–mRNA duplex were visualized using RNAfold[26] and simRNAweb respectively.[5,29]

Lastly, the miRNA–mRNA duplex was docked with AGO proteins (PDB ID: 3F73, chain A) using PatchDock server[13,39]. All analyses were performed under the default parameters of each software[2,27,29,44]. The complete procedure of the study is shown in figure 1.

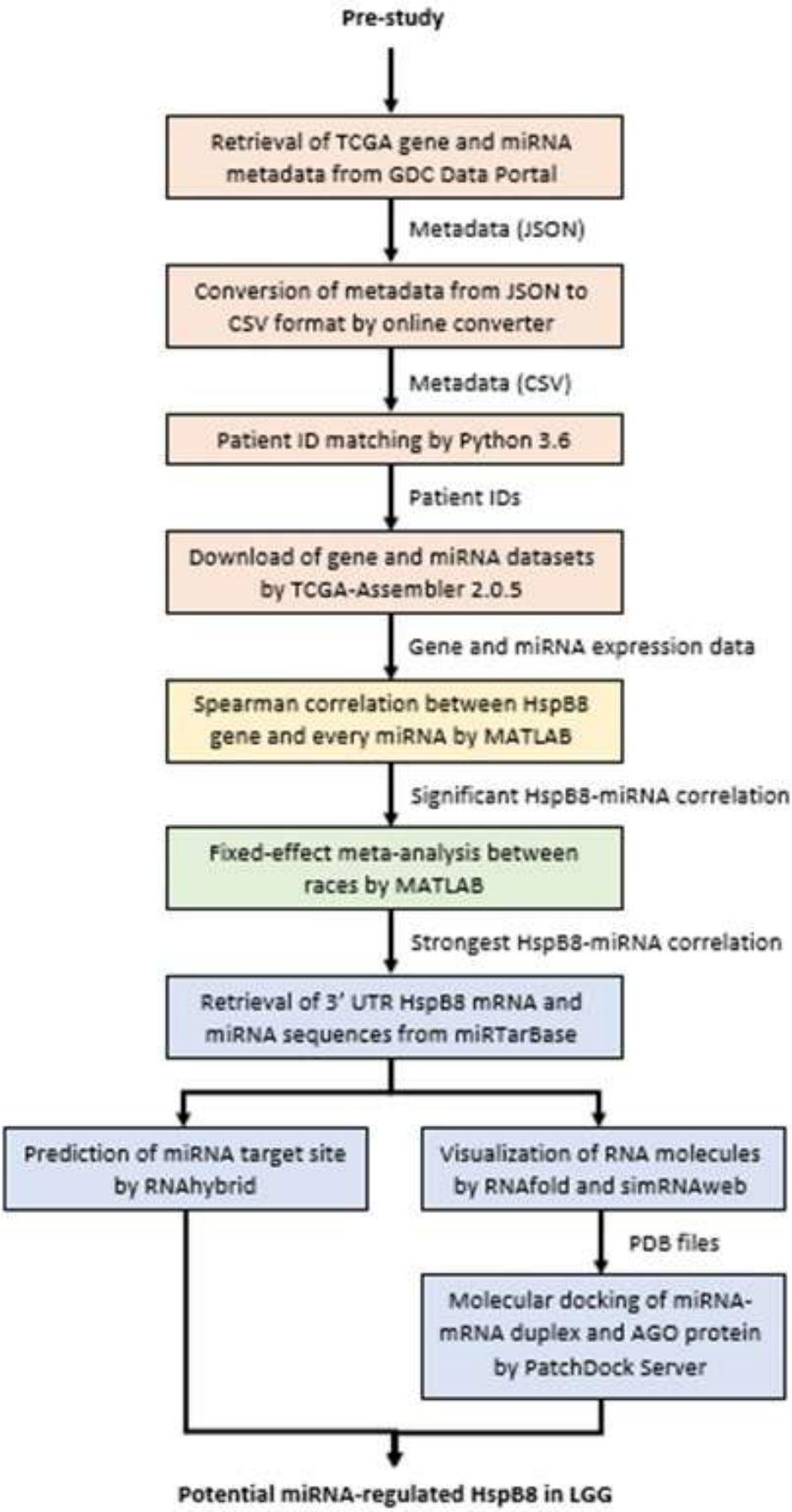

Fig. 1: Flowchart of the procedure, Red: data preprocessing, orange: correlation analysis, green: meta-analysis, blue: transcriptomic validation

Results and Discussion

In this study, the gene and miRNA expression metadata were divided into three groups on the basis of races: white, black or African–American, and others. Such division was performed to ensure that miRNA-regulated HspB8 was found among all LGG patients regardless of their races. The grouping was set on the basis of the assumption that white and black represented two opposite poles of skin colors whereas others were located in between. Initially, 487–488, 21–22, and 19–19 gene-miRNA expression data for white, black, and others were respectively obtained.

After the gene and miRNA metadata were matched, 482, 22, and 18 patient IDs (files) were found among white, black and other samples, respectively. This matching process was essential because the patient must have the gene and miRNA expression data to be included in the correlation analysis. These patient IDs were used as input in the TCGA Assembler which later downloads the gene and miRNA expressions of each patient.

Based on the correlation analysis, we found 20, 47, and 34 significant negative correlations between miRNA and HspB8 that were presented in black, white, and other races respectively. The ten strongest correlations from each race were shown in tables 1, 2 and 3 respectively. As we aimed to propose a miRNA-regulated biomarker that applies for all races, only correlations that appeared in all the races were retrieved (Table 4). The summary correlation value for every association was determined by conducting meta-analysis on the basis of the fixed effects model. This model was selected because the number of samples between groups differed considerably[6]. The results are shown in table 5.

According to table 5, mir-92a-1 had the strongest negative correlation with HspB8. However, this association could not be found in miRTarBase indicating that it is yet to be validated. To assess the feasibility of this interaction, we conducted transcriptomic validation.

After retrieving the sequences of the miR-92a and 3′ UTR of HspB8 from miRTarBase, we predicted the miRNA-target site by using RNAhybrid. On the basis of the result (Figure 2), binding was observed between HspB8 mRNA and miR-92a at the 385th position of the UTR region. This binding was denoted by 14 pairing nucleotides on the second and third lines (yellow-highlighted areas). Nucleotide bindings happen due to the hydrogen bonds between the C–G (three bonds) and A–U (two bonds) of the interacting nucleotides.

Table 1
Top 10 most significant HspB8–miRNA negative correlations in LGG (black and/or African–American Race)

miRNA	Rho Correlation Value	P value
hsa-mir-7112-2	−0.588	<0.05
hsa-mir-9-3	−0.573	<0.05
hsa-mir-93	−0.539	<0.05
hsa-mir-4705	−0.538	<0.05
hsa-mir-6870	−0.516	<0.05
hsa-mir-548f-4	−0.508	<0.05
hsa-mir-6862-1	−0.485	<0.05
hsa-mir-9-1	−0.483	<0.05
hsa-mir-9-2	−0.479	<0.05
hsa-mir-4701	−0.478	<0.05

Table 2
Top 10 most significant HspB8–miRNA negative correlations in LGG (white race)

miRNA	Rho Correlation Value	*P* value
hsa-mir-130b	−0.416	<0.05
hsa-mir-92a-1	−0.403	<0.05
hsa-mir-301b	−0.382	<0.05
hsa-mir-19b-1	−0.341	<0.05
hsa-mir-17	−0.331	<0.05
hsa-mir-4746	−0.314	<0.05
hsa-mir-15b	−0.313	<0.05
hsa-mir-19a	−0.294	<0.05
hsa-mir-16-2	−0.293	<0.05
hsa-mir-19b-2	−0.293	<0.05

Moreover, the minimum free energy of the binding was −23.6 kcal/mol showing a favorable interaction. However, the *p* value was 1.00, which was not significant. Therefore, further analysis was conducted. In addition, the sequences from figure 2 are shown in table 6.

Based on table 6, we predicted the secondary structure of all RNA molecules by using RNAfold. Then, the dot-bracket notations (Table 7) were input into simRNAweb to predict the 3D structure of the RNAs. The secondary and tertiary structures of the RNAs are shown in figures 3 and 4 respectively. Lastly, the miRNA–mRNA duplex molecule was docked with AGO proteins, a significant player in the mRNA silencing process by using the PatchDock server. The first docking model was then retrieved; the statistical analysis and visualization are presented in table 8 and figure 5 respectively.

A previous study showed that Hsp22 (HspB8/Hsp11) exhibits an antiproliferative property in human glioblastoma cells[33]; the knockdown of Hsp22 (HspB8/Hsp11) increases the expression of Sam68 (Src-associated protein in mitosis 68 kDa) and enhances the proliferation of glioblastoma cells. However, based on Firebrowse (http://firebrowse.org/), HspB8 expression in LGGs is the fifth highest among all types of cancer. Generally, the expression of TSG is higher in specific tissues where the repression activity is needed[34] resulting in an opposite effect for oncogenes. If this is the case, then HspB8, instead of TSG, should be an oncogene.

Table 3
Top 10 most significant HspB8–miRNA negative correlations in LGG (other races)

miRNA	Rho Correlation Value	*P* value
hsa-mir-92a-1	−0.725	<0.05
hsa-mir-19b-2	−0.701	<0.05
hsa-mir-20a	−0.695	<0.05
hsa-mir-19a	−0.692	<0.05
hsa-mir-181a-1	−0.686	<0.05
hsa-mir-503	−0.657	<0.05
hsa-mir-4254	−0.634	<0.05
hsa-mir-130b	−0.616	<0.05
hsa-mir-17	−0.612	<0.05
hsa-mir-3140	−0.612	<0.05

Table 4
Significant HspB8–miRNA negative correlations in LGG (all races)

miRNA	Rho Correlation Value			*P* value
	White	*Black*	*Other*	
hsa-mir-92a-1	−0.403	−0.440	−0.725	<0.05
hsa-mir-181b-2	−0.259	−0.434	−0.501	<0.05

Table 5
Results of meta-analysis with 95% CI

miRNA	LL_r[a]	r[b]	UL_r[c]	*P* value
hsa-mir-92a-1	−0.343	−0.417	−0.486	<0.05
hsa-mir-181b-2	−0.192	−0.273	−0.351	<0.05

[a.] Lower limit, [b.] Correlation summary, [c.] Upper limit

Table 6
Sequences of miR-92a and its predicted target site on HSPB8 mRNA

RNA	Sequences
Mature mir-92a-1	AGGUUGGGAUCGGUUGCAAUGCU
Predicted miRNA-target site	ACCAAACCCUCGGUACCCUUAGCCCUCG G
miRNA–mRNA duplex	CGUAACGUUGGCUAGGGUUGGAACCAAA CCCUCGGUACCCUUAGCCCUCGG

Table 7
Secondary structure of RNA molecules

RNA	Dot-bracket Notation	*MFE*[a] (kcal/mol)
Mature mir-92a-1	..(((((.((....)).)))))...	−1.90
Predicted miRNA-target site	......((...(((.......)))...))	−2.10
miRNA–mRNA duplex	.....((..((((((((((....(((........)))))))).))))))..)).	−17.90

[a] Minimum free energy

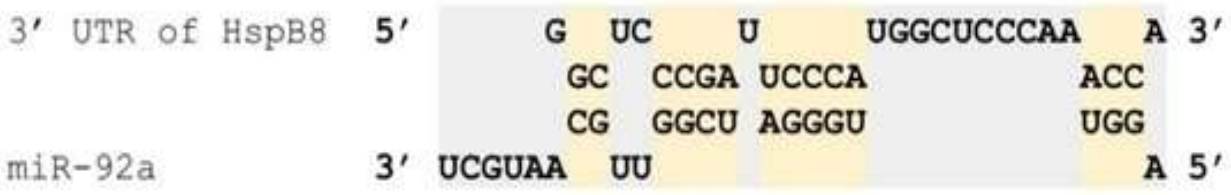

Fig. 2: Prediction of the miR-92a binding site on the 3′ UTR of the HspB8 mRNA—the second line is part of the first line (HspB8), and the third line is part of the fourth line (miR-92a); the interacting nucleotides are placed near each other and are highlighted in yellow; U: uracil, A: adenine, G: guanine, and C: cytosine

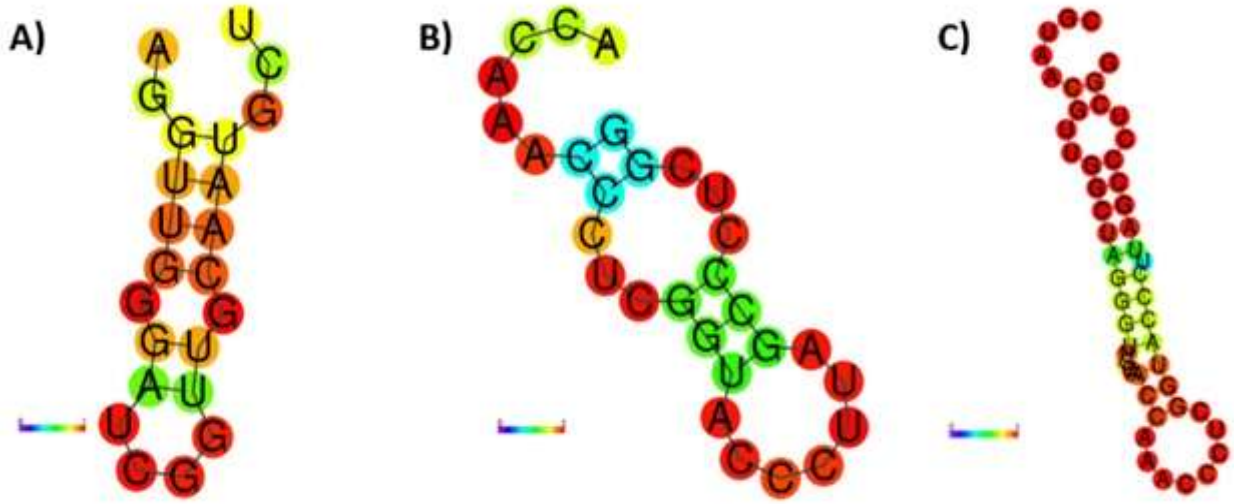

Fig. 3: Secondary structure of RNA molecules—A: miR-92a-1, B: miRNA-target site of HspB8, and C: miRNA–mRNA duplex; the colors denote the conserved region with respect to the structure on the basis of the base-pair probability parameter from 0 (blue) to 1 (red)

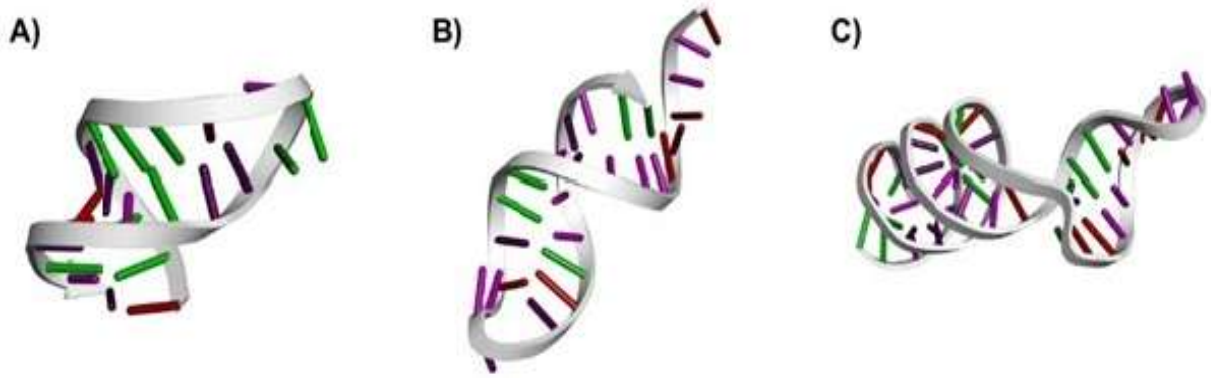

Fig. 4: Tertiary structure of RNA molecules—A: miR-92a-1, B: miRNA-target site of HspB8, and C: miRNA–mRNA duplex; the molecule was visualized using using Discovery Studio Visualizer 2017

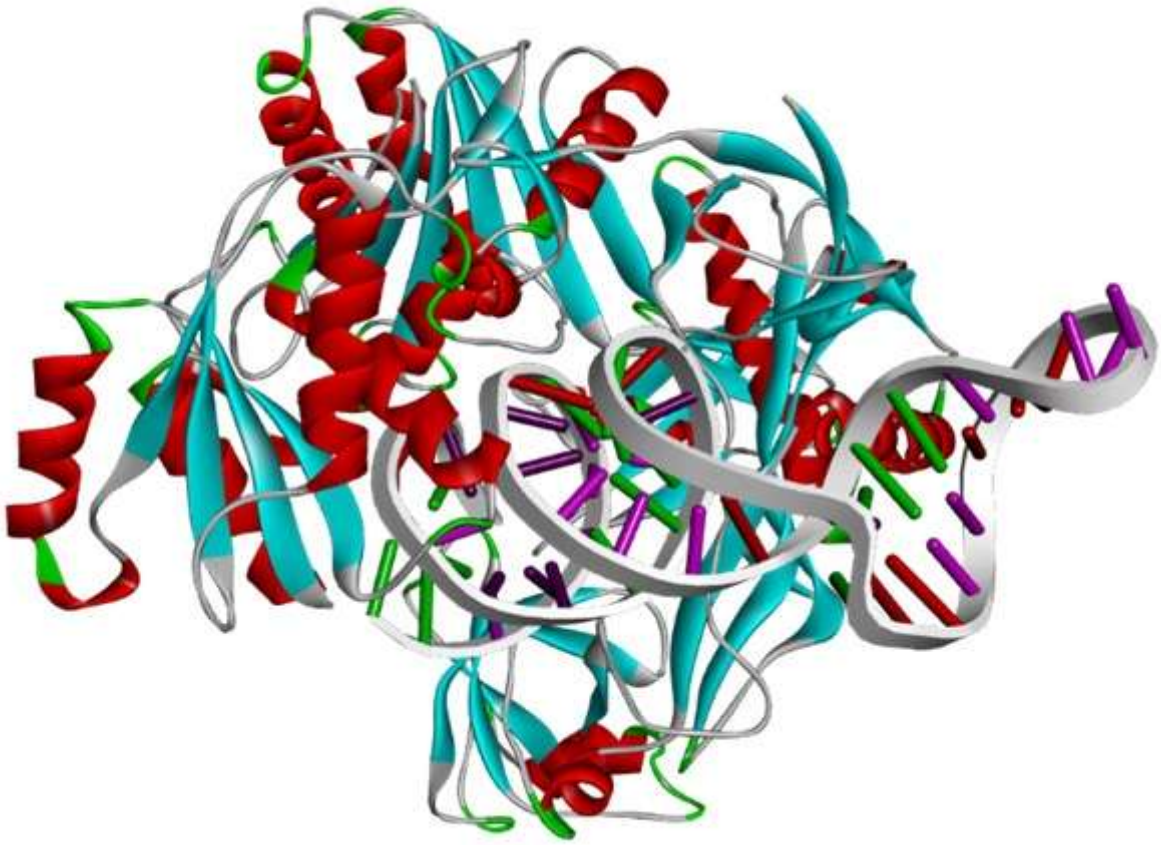

Fig. 5: Docking result between the miRNA–mRNA duplex with AGO protein. The molecule was visualized using Discovery Studio Visualizer

Table 8
Docking scores between miRNA–mRNA duplex with AGO protein

Molecules	Score	Area	*ACE*[a]	Transformation
miR-92a-1 and HspB8-AGO	20956	3219.30	−643.69	1.66 0.69 1.36 16.61 6.30 31.48

[a.] Atomic contact energy

Mature miR-92a regulates human embryonic stem cell differentiation and plays a role in the development of mammalian organs and even in the formation of blood vessels[25]. A previous study showed that miR-92a is overexpressed in glioblastoma cells but lowly expressed in glioma stem-like cells[40]. Glioma stem-like cells are cancer cells found in glioma cells and play an important role in tumor recurrence and sensitivity against treatment[23]. Song et al[40] showed that miR-92a can act as oncogene and TSG. As an oncogene, it downregulates the CDH1/β-catenin signaling pathway, thus increasing the cellular invasiveness and metastatic activity of cancer cells. It also downregulates the expression of notch proteins correlated with cancer metastasis and angiogenesis.

Multiple functions of miR-92a and HspB8 were found; thus, we could not determine the regulation of their interaction in LGGs. Two mechanisms of action are possible because the gene and miRNAs were negatively correlated. These mechanisms are as follows: miR-92a is upregulated and promotes cancer growth whereas HspB8 is downregulated and represses cancer metastasis or the opposite (i.e. miR-92a acts as TSG and HspB8 acts as an oncogene).

The importance of HSP families in cancer development was described by Chatterjee and Burns[9]. In eukaryotes, HSPs primarily act as a molecular chaperone facilitating and maintaining the folding of proteins. Cancer cells often consist of misfolded oncoproteins, thus requiring HSPs to perform the correction. As a result, HSPs are found to be highly expressed in cancer. One of the most studied HSPs is Hsp90, which is correlated with several types of cancer such as lung[12] and medulloblastoma[3]. The inhibition of this particular HSP has produced preclinically promising results[9], meaning that comprehensive HSP studies including those of HspB8, are essential to uncover potent therapeutic treatments for cancer.

In this study, the dataset was limited to the one that is available in TCGA. No data for healthy patients were available. Moreover, the number of samples between groups (races) was significantly different. However, we could not perform meta-analysis based on the random effects model because the samples were dominated by one group[6]. In the future, additional data will be beneficial to strengthen the results.

Conclusion

HspB8 and miR-92a-1 were found to be significantly correlated among LGG patients with an intermediate summary correlation value of −0.417 (p value <0.05). Supported by transcriptome analysis and previous studies, this miRNA-regulated gene provided the basis for a novel potent LGG biomarker for further wet lab studies or for conducting a thorough molecular dynamic simulation in high-performance computers to examine the biochemical reaction mechanism in a fine-grained manner.

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