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GENE & PROTEIN IN DISEASE

Editors-in-Chief

Wei Wang

Edith Cowan University, Australia

Gautam Sethi

National University of Singapore, Singapore



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A graphic illustration of double-stranded DNA

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EDITORIAL**Inaugural editorial for *Gene & Protein in Disease*****Wei Wang^{1†*} and Gautam Sethi^{2†}**¹Center for Precision Health, School of Medical and Health Sciences, Edith Cowan University, Joondalup, Australia²Department of Pharmacology, National University of Singapore, Singapore

With our immense pleasure, humility, and expectation, we celebrate the launch of *Gene & Protein in Disease* (GPD) with this inaugural issue. Together with Dr. Xinying Ji, the Managing Editor, and the entire Editorial Team, we are honored and privileged to extend a very warm welcome to the readership of GPD. We would like to appreciate the valuable contributions from our prospective authors, editors, anonymous reviewers, and AccScience Publishing, which have made the launch of GPD possible.

GPD primarily covers research on the complex interplay between genes, environments, and behavior utilizing multiple levels of analysis, for example, genomics, proteomics, transcriptomics, glycomics, lipidomics, metabolomics, and microbiome study, with advanced techniques both *in vivo* and *in vitro*, for example, genetics, epigenetics, genetic editing, molecular, cellular, tissue, animal model, and bioinformatics approaches.

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[†]Both Professor Wei Wang and Professor Gautam Sethi are Editors-in-Chief of *Gene & Protein in Disease*

***Corresponding author:**
Wei Wang (wei.wang@ecu.edu.au)

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Conflict of interest

No conflict of interest has been declared by the authors.

ORIGINAL RESEARCH ARTICLE

Structural, functional, phylogenetic, and molecular dynamic simulation study of PEST-containing nuclear protein: An e-science view

**Nazeer Hussain Khan^{1,2}, Muhammad Shahid³, Wenkang Wang⁴,
Saadullah Khatak^{1,2}, Ebenezeri Erasto Ngowi^{1,5}, Salma S. Mahmoud^{1,6},
Hao-Jie Chen¹, Lei Qian¹, Yangzhe Qin¹, Tao Li¹, Muhammad Zubair⁷,
Shazrul Fazry³, Dong-Dong Wu^{1,8*}, Chun Yang Zhang^{9,10*}, and Xin-Ying Ji^{1,11*}**

¹Henan International Joint Laboratory for Nuclear Protein Regulation, School of Basic Medical Sciences, Henan University, Kaifeng, Henan, China

²School of Life Sciences, Henan University, Kaifeng, Henan, China

³Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Selangor, Malaysia

⁴Department of Breast Surgery, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan, China

⁵Department of Biological Sciences, Faculty of Science, Dar es Salaam University College of Education, Dar es Salaam, Tanzania

⁶Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Ain Shams University, Cairo, Egypt

⁷Department of Wildlife and Ecology, University of Veterinary and Animal Sciences, Pattoki Campus, Pakistan

⁸School of Stomatology, Henan University, Kaifeng, Henan, China

⁹Department of Thoracic Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, P.R. China

¹⁰Department of General Thoracic Surgery, Hami Central Hospital, Hami, Xinjiang, P.R. China - Institute of Nursing and Health, Henan University, Kaifeng, Henan, China

¹¹Kaifeng Key Laboratory of Infection and Biological Safety, Henan University College of Medicine, Kaifeng, Henan, China

*Corresponding authors:

Dong-Dong Wu
(ddwubiomed2010@163.com)
Chun Yang Zhang
(zcy198200@163.com)
Xin-Ying Ji
(10190096@vip.henu.edu.cn)

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Abstract

PEST-containing nuclear protein (PCNP) is a short-lived novel nuclear protein. It has been well evaluated that PCNP mediates the progression of several cancers, but the exact mechanisms are still under investigation. In this study, we provided an e-science view of PCNP protein from the aspects of protein structure, interactions, and bioinformatics-based analysis related to evolutionary features as well as proteomic profile. The phylogenetic relationship results reveal that PCNP is closely related to Pan troglodytes and the Bovidae family, while being distantly related to the Muridae family. The analysis of the physicochemical properties of PCNP demonstrated that it is a thermolabile protein which is slightly acidic and hydrophilic in nature. Further, coexpression and protein-protein interaction analyses were carried out, which demonstrated that the PCNP gene was remarkably expressed with *MORF4L1* and *RSL24D1* genes and has close interactions with TRAM1, PSMC6, SRP9, PRKRIR, UHRF2, and BMI1 proteins. Gene ontology and pathway enrichment analyses showed that PCNP has a high tendency to work in cell cycle regulation. Moreover, among the four 3D structure generating tools, I-TASSER-generated structure had the highest

quality factor score. The validation analysis revealed that the I-TASSER-generated structure exhibited the best quality factor score with maximum amino acids in the favored region. In addition, molecular dynamic simulation analysis approved the stable structure of the PCNP. This is the first study that highlights the usefulness of the understanding of the structural and functional analysis of the PCNP, which lays the groundwork for further experimental studies to validate the outcome.

Keywords: 3D structure; Molecular dynamic simulation; PEST-containing nuclear protein; Phylogeny; Physiochemical properties; Protein interactions

1. Introduction

Nuclear proteins (NPs) play a key role in the continuity of cell life by regulating the cell cycle and maintaining the complex organization of the cell^[1]. There are various known families of NPs that play very essential roles in various cellular functions. With unique structure and functions, a family of NPs known as PEST-proteins (PEST-NPs), which are rich in proline (P), glutamic acid (E), serine (S), and threonine (T) sequences, acts as signal peptides for protein degradation^[2,3]. PEST-NPs are also regarded as the guardians of the cell and are believed to play crucial roles in cell cycle regulation, ubiquitin proteasome pathway, glycosylation of nuclear pores, hexosamine biosynthetic pathways, cell nutrient uptake and cellular metabolism regulation, and nucleocytoplasmic transport^[4-6]. It has been well reported that PEST sequences serve as a proteolytic signal to degrade the target proteins through proteasome or calpain proteolysis pathways^[7,8]. In 2002, Mori *et al.* discovered a novel PEST-containing nuclear protein (PCNP), which is involved in vital cellular processes such as cell proliferation and mediates tumorigenesis. It is a short-living and small NP of only 178 amino acids with two remarkable PEST sequences^[9]. Since its discovery, its role has been studied in several cancers for its relation with cancer progression, and now, it is recognized as an oncogenic protein.

PCNP has not been fully explored yet. There are very few molecular and genomic profiling studies regarding PCNP expression in cancer cells. However, in our previous *in vitro* and *in vivo* studies, we have exploited the tumorigenic roles of PCNP in neuroblastoma, lungs, and ovarian cancers^[10-12]. It has been found that PCNP is upregulated in human lung adenocarcinoma, and mediates the proliferation, migration, and invasion of cells. However, knockdown of the *PCNP* gene is able to reverse the result and retard the progression of adenocarcinoma in the lungs^[10]. In another study, we evaluated the PCNP gene expression in neuroblastoma using the SH-SY5Y and SK-N-SH cell lines. The findings showed that compared to the normal control group, the high expression of PCNP decreased the growth, migration, and invasion of human neuroblastoma

through MAPK and PI3K/AKT/mTOR signaling pathways and promoted the apoptosis of neuroblastoma. Meanwhile, we also observed opposite results after silencing the *PCNP* gene in human neuroblastoma cells. These results were further strengthened by xenograft tumor analysis in animal experiments which employed both PCNP overexpression and knockdown approaches^[11]. Recently, we also revealed a novel association of PCNP with the Wnt pathway^[12]. PCNP is overexpressed in both ovarian cancer tissues and cells than in para-cancerous tissues and ovarian epithelial cells (IOSE80). Through *in vitro* and *in vivo* experiments on ovarian cancer cells (SK-OV-3 and A2780), it has been found that high expression of PCNP promotes growth, migration, and invasion of ovarian cancer cells and inhibits their apoptosis. Furthermore, PCNP could bind to β -catenin, promote β -catenin nuclear translocation, and then activate the Wnt/ β -catenin signal pathway^[12]. Based on all these findings, we justify that PCNP can be a promising biomarker for diagnosis and prognosis of cancer. Moreover, a potent PCNP inhibitor strategy could be adopted for exploring PCNP's role in tumorigenesis.

Therefore, it is essential to explore the mechanism of PCNP in cancer progression and its applicability as a potential target in cancer diagnosis and treatment. To the best of our knowledge, there has been no reported study on PCNP structure, interactions, and other bioinformatics analysis related to its evolutionary features as well as proteomic profile, and this is the first study to present the three-dimensional (3D) structural modeling, interaction with other proteins, and physiochemical properties of PCNP. The *in silico* analysis performed in this study demonstrated the features and properties of PCNP collected from different online web servers and software, which help increase our understanding of PCNP and its functions in cellular activities in tumorigenesis.

2. Materials and methods

2.1. Data retrieval and multiple sequence alignment

The protein ID and FASTA amino acid sequence of PCNP were retrieved from the National Center for Biotechnology

Information (NCBI) (<https://www.ncbi.nlm.nih.gov/>) with accession reference NP_065090.1. To accomplish this study, we employed various bioinformatics tools and software as listed in Table 1.

2.2. 3D structure prediction

PCNP has no reported structure in the protein data bank (PDB) database (<https://www.rcsb.org/>)^[13]. Therefore, to generate the 3D structure, both protein comparative homology and sequence-based structure modeling approaches were utilized. We adopted four DeepMind learning tools that predict the protein structures, for example, AlphaFold (<https://alphafold.ebi.ac.uk/>), RoseTTAFold (<https://boinc.bakerlab.org/rosetta/>), Iterative Threading Assembly Refinement (I-TASSER) (<https://zhanggroup.org/I-TASSER/>), and Robetta (<https://rosetta.bakerlab.org/>)^[14-17]. AlphaFold and RoseTTAFold are new computational tools that use deep learning algorithms to generate protein structures. Besides, they predict the protein structures in cases where no homology structure is known by incorporating the limited physical and biological knowledge into the deep learning algorithms^[18,19]. I-TASSER and Robetta are publicly available web-based tools that predict 3D protein structure. They use the amino acid sequence as input and generate the 3D structure either by searching for multiple threading alignments for the template from the PDB or using iterative structural assembly simulations, a *de novo* structure prediction method^[16,17]. Graphical inspection and representation of the models were done using UCSF Chimera software version 1.14 (<http://www.cgl.ucsf.edu/chimera/>)^[20].

Furthermore, the resultant structures from the aforementioned tools were further subjected to the ERRAT and PROCHECK tools. Both are in-built tools of the Structure Analysis and Verification Server (SAVES) meta-server version 6.0 (<https://saves.mbi.ucla.edu/>) that determine the quality factors (degree of appropriateness) and stereochemical evaluation of the query protein structure^[21,22]. In addition, the quality model energy analysis (QMEAN) was performed using the QMEAN server accessed from <https://swissmodel.expasy.org/qmean/>. It is a composite scoring function that estimates the quality of a protein structure model by deriving the local (per residue) or global (for the entire protein structure) scores in terms of a Z-score^[23].

2.3. Molecular dynamic simulation (MDS)

MDS of the best scored PCNP protein structure from the above-mentioned tools was carried out using GROMACS software^[24]. In *in silico* studies, MDS is considered the key analysis to identify the interactions of protein molecules and their structural characteristics^[25]. For MDS run, the PCNP

Table 1. List of various bioinformatics tools employed in the present study.

S. No.	Database/tool	Functions
1.	National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/)	To retrieve amino acid sequence of protein
2.	Protein Data Bank (https://www.rcsb.org/)	Structure of protein
3.	AlphaFold (https://alphafold.ebi.ac.uk/)	Protein structure construction
4.	RoseTTAFold (https://boinc.bakerlab.org/rosetta/)	Protein structure construction
5.	I-TASSER (Iterative Threading Assembly Refinement; https://zhanggroup.org/I-TASSER/)	Protein structure construction
6.	Robetta (https://rosetta.bakerlab.org/)	Protein structure construction
7.	UCSF Chimera software version 1.14	Graphical inspection and representation of the models
8.	SAVES meta-server version 6.0 (https://saves.mbi.ucla.edu/)	To determine the quality factors of protein structure
9.	Quality Model Energy Analysis (QMEAN; https://swissmodel.expasy.org/qmean/)	Quality of a protein structure by deriving the local or global scores in terms of Z-score
10.	GROMACS software	Molecular dynamic simulation of protein structure
11.	ProtParam tool of ExPASy (http://web.expasy.org/protparam/)	To determine the basic physicochemical properties
12.	SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html)	For the secondary structure prediction of a protein
13.	GeneMANIA server (https://genemania.org/)	To check the co-expression analysis of a protein
14.	STRING protein database (https://string-db.org/)	PPI of protein
15.	Cytoscape software version 3.8.2	To construct the coexpression and PPI networks
16.	Network-Analyst server (https://www.networkanalyst.ca/)	To assimilate the biological processes, molecular functions and cellular components, and to enrich pathway analysis of protein
17.	Enrichr web-server (https://maayanlab.cloud/Enrichr/)	To validate the Network Analyst results
18.	Molecular Evolutionary Genetic Analysis (MEGA Home; megasoftware.net)	To construct phylogenetic tree of gene
19.	Sequence Demarcation Tool Version 1.2 (SDTv1.2; http://web.cbio.uct.ac.za/~brejnev/)	For pairwise sequence identity of a gene in evolutionary analysis
20.	Google Scholar	To retrieve the literature

PPI: Protein-protein interaction

protein structure was prepared using the GROMOS96 43a1 force field solvated by the single point charge water model and was fixed in a periodic cubic solvated box. Other parameters were neutralized by adding the salt (NaCl) concentration (0.15 M), energy minimization for 5000 steps of the prepared system, and equilibration type NVT/NPT at 300 K and 1 bar of pressure. The final MDS step was processed for 50 ns of time. The MDS was analyzed by calculating the root mean square deviation (RMSD) and root mean square fluctuation (RMSF) values of the protein structure.

2.4. Physicochemical characterization and secondary structure prediction

The basic physicochemical properties, such as theoretical isoelectric point (pI), molecular weight, total number of positive and negative residues, extinction coefficient instability index, aliphatic index, and grand average hydropathy, were computed using the ProtParam tool of ExPASy (<http://web.expasy.org/protparam/>)^[26-28]. For the secondary structure prediction of PCNP, SOPMA bioinformatics tool (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) was used with its default query parameters^[29]. This tool uses the self-optimized prediction method and a neural network method (PHD) to predict the secondary structure of proteins^[29,30].

2.5. Coexpression and protein-protein interaction (PPI) analysis

The coexpression analysis of the PCNP was performed by the GeneMANIA server (<https://genemania.org/>) by setting *Homo sapiens* as the species organism and the maximum number of resultant genes and attributes as 10. It is a web interface that has a high-accuracy prediction algorithm and a large database for gene functional analysis, including coexpression, genetic interaction, colocalization, physical interaction, pathway interaction, and shared protein domains of the submitted query^[31]. Furthermore, the PPI of PCNP was evaluated by accessing the search tool for the retrieval of interacting genes (STRING) protein database (<https://string-db.org/>). The PPI network was constructed based on the data from tandem affinity purification assay, affinity chromatography technology assay, and coimmunoprecipitation assay. The STRING database query terms were cutoff at 0.4 and the maximum additional interaction was 10^[32]. Cytoscape software version 3.8.2 (<https://cytoscape.org/>) was employed to construct the coexpression and PPI networks^[33].

2.6. Gene ontology (GO) and pathway enrichment analysis

To assimilate the biological data of GO, including biological processes, molecular functions, and cellular components,

and enriched pathway analysis of PCNP, the Network Analyst server (<https://www.networkanalyst.ca/>) was employed^[34,35]. It is a publicly available online tool widely used for gene expression profiling, transcriptional factor analysis, PPI networks, pathway analysis, toxicogenomics, and pharmacogenomics studies^[36,37]. In addition, the Enrichr web server (<https://maayanlab.cloud/Enrichr/>) was utilized to validate the Network Analyst results^[35].

2.7. Phylogenetic analysis

To determine the evolutionary history of PCNP between humans and other vertebrates, the phylogenetic tool, molecular evolutionary genetics analysis (MEGA) X, was used^[38]. PCNP has no reported paralogs in the biological database (ENSEMBL) or in the available literature. Orthologs of PCNP were used for tree construction and sequences of eight orthologous candidate proteins were retrieved from the NCBI database. Alignment of multiple sequences was carried out using the MUSCLE program implemented within MEGA X. For pairwise sequence identity, Sequence Demarcation Tool Version 1.2 (SDTv1.2; <http://web.cbio.uct.ac.za/~brejnev/>) was used^[39]. The best-fit substitution model was estimated in MEGA and selection was done based on the Neighbor-Joining Method. The rate of variation among sites was modeled with a gamma distribution (shape parameter = 1). All positions that contained alignment gaps and missing data were eliminated from the analysis. A phylogenetic tree was constructed using the unweighted pair group method with arithmetic mean, and the validity of the tree generated was tested by bootstrap analysis of 1000 pseudoreplicates^[40,41].

3. Results and discussion

3.1. Structure prediction

Four tools were utilized to generate the 3D structure of the PCNP protein. From the results of all these tools, we selected the best score-generating model and subjected it to further validation through the ERRAT and PROCHECK servers. In addition, the quality model QMEAN score was evaluated for the best scoring models. The graphical illustration of these four best models is presented in Figure 1. Following the 3D model analysis, it was discovered that the I-TASSER generated model had the highest quality factor score among the four best-scoring models, as shown in Figure 1D and Table 2. Moreover, the I-TASSER model structure was subjected to MDS for profound validation analysis.

3.2. Molecular dynamics simulation

An MDS was conducted for the analysis of the stability and flexibility of the I-TASSER-generated PCNP protein model. The RMSD and RMSF of the protein structure

were calculated using MDS for the 50-ns time interval. The results of RMSD and RMSF are presented in Figure 2. The RMSD values were observed in the range of 0.2–0.9 nm of the protein model. In brief, the results revealed that the RMSD value came to equilibrium, maintaining around 0.6–0.8 nm throughout the MDS run of 50 ns (Figure 2A). Although the lack of experimental structure, these MDS results suggest that the protein model is dynamically stable throughout the MDS period with a slightly higher RMSD value. Similarly, the amino acid residue wise fluctuation, the RMSF value, was plotted and is shown in Figure 2B. The RMSF analysis demonstrated that notable deviations have occurred in the first 20–25 amino acid residues, but this type of high fluctuation was not observed in other residues throughout the MDS period.

3.3. Physicochemical characterization and secondary structure prediction

The ProtParam tool, available on the ExPASy server, predicts the physicochemical properties of the protein under investigation. The PCNP gene encodes a protein of 178 amino acids ($C_{805}H_{1304}N_{238}O_{280}S_4$) having a molecular weight of 18924.88 kDa. The theoretical isoelectric point (pI) of this protein was found to be 6.86 with 28 negatively charged residues (Asp + Glu) and 28 positively charged residues (Arg + Lys). At this computed pI, the PCNP protein seemed to be stable and compact and was considered slightly acidic. These results will be helpful in preparing

a buffer system for purification by the pI method^[42]. The instability index of PCNP protein was computed as 15.56, which indicates that the protein may be marginally stable as the values are lower than 40. The instability index of a protein greater than 40 indicates that the protein is likely to be unstable.

In addition, the value of the aliphatic index, which is the relative volume of aliphatic side chains, indicates the thermal stability of a protein structure. The higher the positive factor value, the greater the thermal stability of the respective protein. The aliphatic index of PCNP was calculated to be 89.93, indicating its stability at higher temperatures^[43]. The grand average of the hydropathicity of the protein was predicted to be −0.463, which indicates the hydrophilic nature of the protein and improved interaction with water. The detailed results are documented in Figure S1.

Of the total 178 amino acids, 58 (32.58%) were found in the alpha helix, 11 (6.18%) in the extended strand, 8 (4.49%) in the beta turns, and 101 (56.74%) in the random coil region of the PCNP protein as predicted by the SOPMA tool. These results demonstrate that the PCNP protein secondary structure has a dominant random coil region followed by beta turns and an alpha helix. The detailed results are presented in Figure S2.

3.4. GO and pathway enrichment analysis

GO is an efficient machine learning method useful for the identification of biological processes, molecular functions, and cellular components of a query protein. In the present study, a total of 46 biological processes, 10 molecular functions, and 10 cellular components of PCNP were identified by the Network Analyst server (Table S1). All the predicted biological processes results were below 0.05 (*P*-value) and considered a significant outcome. The top 10 biological processes results are demonstrated in Figure 3A. In biological processes analysis, our results indicate that PCNP has proximate and profound involvement in essential cellular activities, such as cell cycle, cycle arrest, and cellular catabolic processes (Table S1). Out of the 10

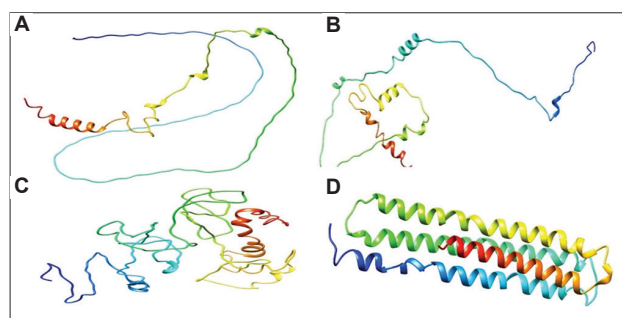


Figure 1. The 3D structures of PCNP protein predicted by (A) AlphaFold, (B) RoseTTAFold, (C) Robetta servers, and (D) I-TASSER.

Table 2. The quality factors-score values of four structures of PCNP by predicted by various corresponding servers.

Protein	SERVER	SWISS MODEL		PROCHECK			PROSAWEB	ERRAT
		QMEAN4 Score	QMEANDisCo Global Score	Favored region (%)	Allowed region (%)	Disallowed region (%)	Z-score	ERRAT
PCNP	1 AlphaFold2	−9.33	0.34±0.06	61.5	37.8	0.7	−1.71	92.4528
	2 RoseTTAFold	−2.39	0.42±0.06	80.4	17.6	2	NA	91.3386
	3 I-TASSER	−6.37	0.28±0.06	87.2	11.5	1.4	NA	94.7059
	4 Robetta	−12.32	0.31±0.05	40.4	55.7	3.8	−3.08	82.8244

Based on these results, I-TASSER model was selected as best structure and subjected to further MDS analysis.

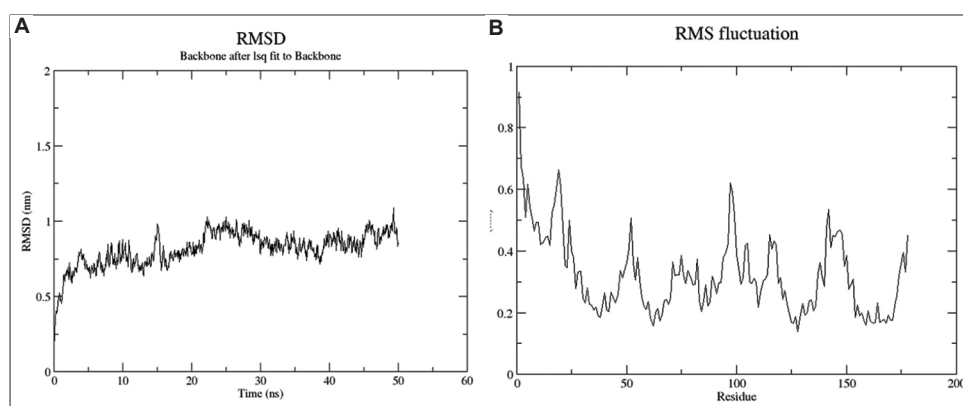


Figure 2. Molecular dynamic simulation of coordinate (A) root mean square deviation and (B) root mean square fluctuations of PCNP structure generated by I-TASSER.

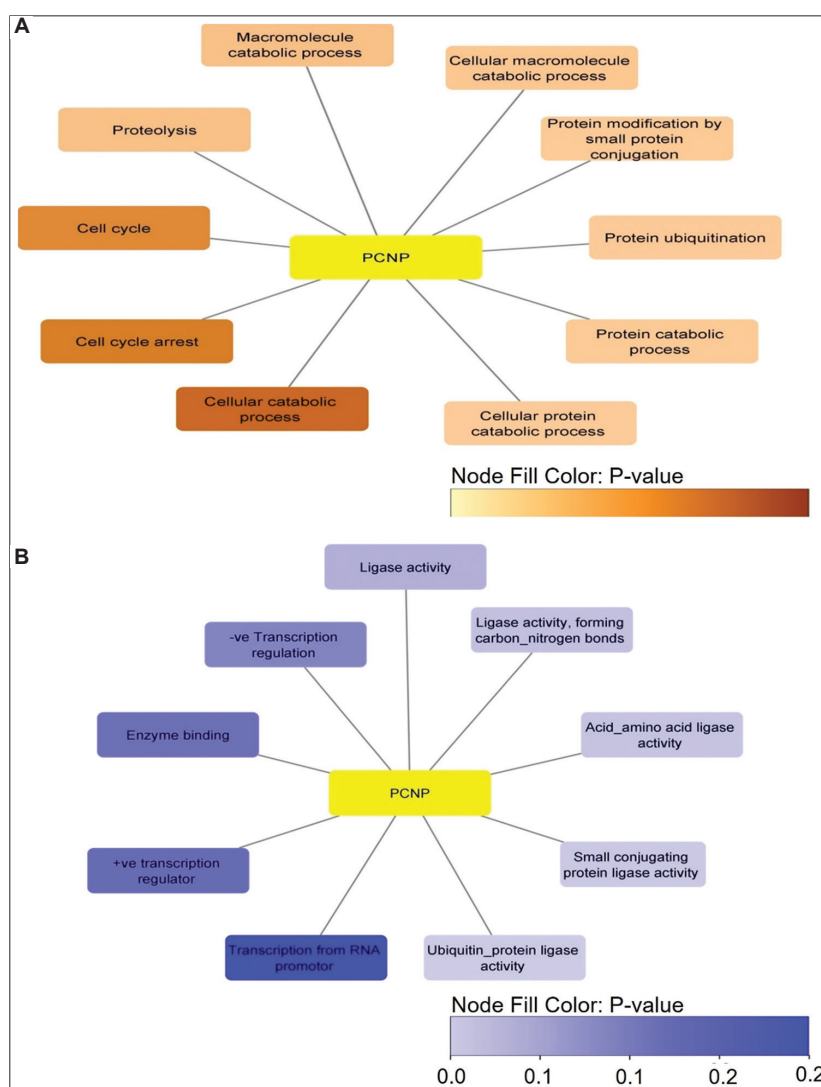


Figure 3. Gene ontology network of (A) the top 10 enriched biological processes and (B) the 10 enriched molecular functions of PCNP constructed by Cytoscape software. In the constructed networks, nodes are color-shaded according to their P-value score i.e., red > orange > yellow for biological processes, and dark blue > blue > light blue for molecular functions.

molecular functions, ubiquitin protein ligase activity, small conjugating protein ligase activity, amino acid ligase activity, and ligase activity forming carbon nitrogen bonds showed $P < 0.05$. The network of molecular functions constructed by Cytoscape software is presented in Figure 3B. Similarly, the cellular components results show the distribution of PCNP solely in the nucleus of the cell (Table S1). Furthermore, the pathway enrichment analysis was performed using the Network Analyst server. There were no results for the PCNP protein when searching broad queries on both servers (Network Analyst and Enrichr) for the Kyoto Encyclopedia of Genes and Genomes (KEGG) Reactome, and WikiPathways.

3.5. Coexpression and PPI analysis

Co-expression of PCNP was investigated by GeneMANIA server. The outcomes of GeneMANIA were subjected to Cytoscape software to construct the network based on their coexpression score. The coexpression results reveal that PCNP has significant expression with *MORF4L1* and *RSL24D1* genes, and the least association of coexpression with *ATF1*, *NFYB*, and *CMPK1* genes (Figure 4). *MORF4* (mortality factor on chromosome 4) is a senescence-inducing gene in tumor cells. The previous research has demonstrated that MORF4 protein is found both in the cytoplasm and the nucleus of cells^[44]. In addition, *RSL24D1* is a NP involved in ribosomal biogenesis^[45]. Hence, these findings are the first to confirm that PCNP is a NP, and it might be a potent protein that plays a role in the regulation of tumor cell progression and ribosomal biogenesis through influencing the expression of *MORF4* and *RSL24D1* genes.

Moreover, in the present study, the PPI was determined based on the data of tandem affinity purification assay, affinity chromatography technology assay, and coimmunoprecipitation assay. From the results, four proteins, namely, TRAM1, PSMC6, SRP9, and PRKRIR, were extracted using tandem affinity purification and coimmunoprecipitation assays. Using the affinity chromatography technology assay, two proteins, UHRF2 and BMI1, were found to have an interaction with PCNP protein (Figure 5). TRAM1 (Translocating chain-associated membrane protein 1) and SRP9 (Signal Recognition Particle 9) are involved in the translocation of nascent secretory proteins by facilitating proper chain folding into or through the endoplasmic reticulum membrane^[46]. To this end, it can be speculated that PCNP might be associated with endoplasmic reticulum protein transport by making direct or indirect interaction with TRAM1 or SRP9. In addition, PSMC6 is located in the base region of the proteasome 19S regulatory particle and is involved in the translocation of the substrates to the core particle. A recent study reported that

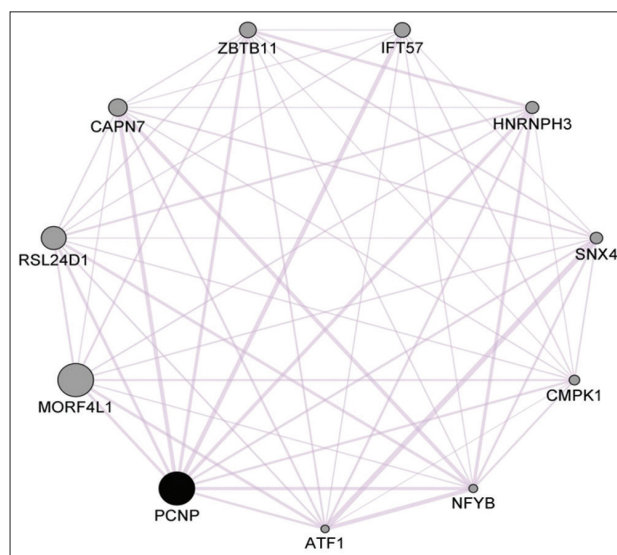


Figure 4. Coexpression network of 10 common targets of PCNP constructed with Cytoscape software. In the network, the size of the node corresponds to the coexpression affinity level with the PCNP. The bigger the size of the node, the greater the coexpression level with the respective gene.

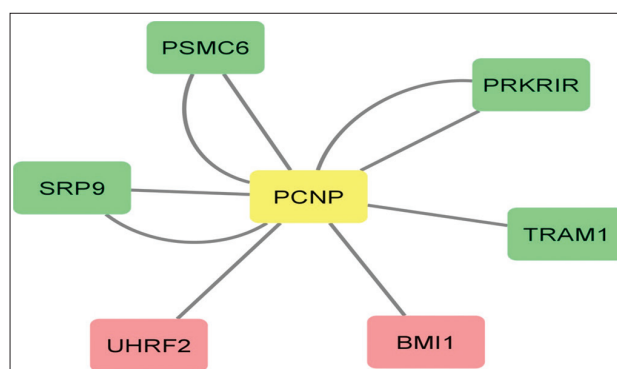


Figure 5. The protein-protein interaction network of PCNP protein based on the experimental models constructed with Cytoscape software. The proteins in the green color nodes were collected from the tandem affinity purification assay and coimmunoprecipitation assays, and the proteins in the pink color nodes were filtered from the affinity chromatography technology assay.

PSMC6 promotes the apoptosis of osteoblasts by halting the PI3K/AKT signal transduction pathway. Interestingly, a previous research study demonstrated PCNP activity through the PI3K/AKT signal transduction pathway. Therefore, these results suggest that the cross-talk between PCNP and PSMC6 is needed to regulate vital functions of the cell through the PI3K/AKT signal transduction pathway^[47]. Moreover, UHRF2 is also a NP involved in cell cycle regulation. It halts cell proliferation and acts as a tumor suppressor factor. BMI1 is a member of polycomb-repressive complex 1 that is necessary for chromatin remodeling or silencing. Importantly, it also plays a role in the self-renewal of somatic stem cells^[48]. Apart from these PPI findings,

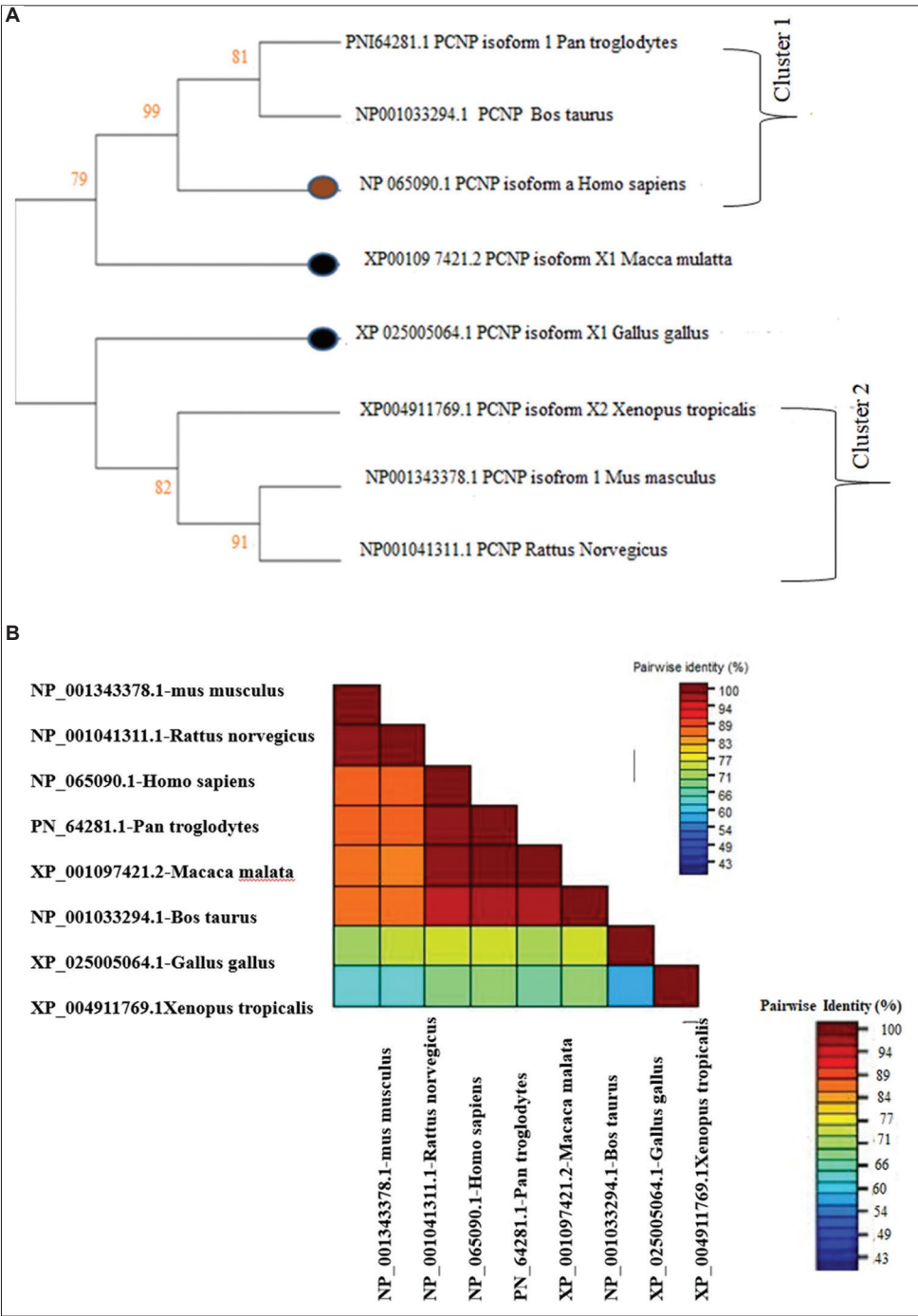


Figure 6. (A) Phylogenetic tree of PCNP) of human and other mammalian species. The tree was constructed using MEGA7 software by the Neighbor-Joining method. Different mammalian species along with GenPept IDs are shown. Numbers along the branches refer to the bootstrap value (percentage of confidence). PCNP sequence (NP 065090) used in this study is highlighted with red solid circle and out-grouped sequence is represented with black solid circle. (B) Pair-wise identity matrix of PCNP sequence of various mammalian species. A color-coded pair-wise identity matrix was generated from PCNP protein sequences (species name and GenPept IDs are shown) using SDT program. Each colored cell represents a percentage of identity score between two sequences (one indicated horizontally while the other vertically). Species forming different subgroups are demarcated by red box.

published literature on coexpression of PCNP reveals that dolichol-phosphate mannosyltransferase subunit 1 (DPM1), a mannose donor in various glycosylation reactions and a

catalytic subunit of the polyol-mannose phosphate (DPM) synthesis complex, is coexpressed with PCNP at the post-translational modification (PTM) level in patients with

congenital disorders^[49]. PTMs refer to amino acid side chain modifications in some proteins after their biosynthesis. Disruption in PTMs can cause malfunctioning of vital biological processes and, hence, lead to various diseases^[50]. Therefore, considering the DPM1 and PCNP coexpression as synchronization of PCNP functions, there is an urgent need to predict PTMs in this least explored protein, which will provide a clear picture of PCNP functions, especially in cancer onset. Based on these already published findings and the predicted outcomes of the present study, it can be deduced that PCNP might have diverse functions, although many of which remain unreported.

3.6. Phylogenetic analysis

The phylogenetic neighbor-joining tree presented in this study reveals many interesting characteristics of the candidate protein in vertebrates. The human PCNP protein phylogenetic tree revealed remarkable similarities to *Pan troglodytes* and *Bos taurus*. The results indicate a close relationship with both species but that they have ancestor linkage in a distinguished clade. The generated phylogenetic tree of PCNP is presented in Figure 6A. Besides, the evolutionary tree shows that the ancestry of PCNP protein is closer to the Bovidae family but far away from other primates like *Mus musculus*. The tree also shows that in the PCNP phylogeny, *Gallus gallus* is an out-group. Moreover, based on the pairwise sequence identity, the PCNP protein from the Bovidae family could be clustered as one subgroup. The pair-wise identity matrix of PCNP sequences and its eight orthologs in various vertebrates is demarcated in Figure 6B. These findings additionally support the phylogenetic tree that presents an indication of the molecular kinship of PCNP proteins belonging to diverse mammalian species. Overall, the *in silico* results reveal many hidden functional, structural, and evolutionary characteristics of the human PCNP protein.

3.7. Scope and limitations of the study

Doubtlessly, the *in silico* study presented here provides a critical, quick, and low-cost structure prediction and functional elucidation of PCNP, and it will add more literature and understanding of PCNP for the future studies. However, there are some limitations to this study, such as the fact that it is a computational study. A study based on the results that were retrieved from public data-tools has no affirmation from a wet laboratory study. Results generated from software implementation of the data have high false positive or false negative rates, which restrict us from being fully confident in the results. Furthermore, PCNP is the least explored protein, so there is a lack of data to validate the molecular dynamics experiments and structural stability. Therefore, it is highly

recommended to perform functional assays in cell lines as well as crystallographic analysis to fully understand the PCNP structure and its binding abilities.

4. Conclusion

Our study presents an *in silico* structural and functional interpretation of the PCNP by employing various bioinformatics tools. We identified the phylogenetic relationship of PCNP and then performed structural and functional analysis. Overall, PCNP is slightly acidic, hydrophilic in nature, and a thermostable protein. The protein secondary structure has a dominant random coil region followed by beta turns and an alpha helix. Through the coexpression and PPI analyses, we discovered that the PCNP gene is expressed with *MORF4LI* and *RSL24D1* genes and has close interactions with TRAM1, PSMC6, SRP9, PRKRIR, UHRF2, and BMI1 proteins. The GO results are consistent with the previously reported studies and endorse PCNP's involvement in vital cell functions, such as cell cycle and cancer progression as an oncogenic protein. Among the four different 3D structure generation tools, I-TASSER generated a model that had the highest quality factor score, which was further validated through MDS. To date, this is the first comprehensive study to elucidate the structural outlook and functional details of PCNP. This computational work, therefore, provides an essential perspective of PCNP, which can be useful for proteomics or genomic research involving extensive *in vivo* experimental work on this vital NP, and helps deepen our understanding regarding the function of PCNP in the nucleus and its potential role as a diagnostic and therapeutic target in cancer biology discipline.

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Conflict of interest

All the authors declare no conflict of interest.

Author contributions

Conceptualization: Nazeer Hussain Khan and Wenkang Wang

Data curation: Nazeer Hussain Khan and Wenkang Wang

Formal analysis: Nazeer Hussain Khan, Wenkang Wang, Muhammad Shahid, Saadullah Khatak, and Ebenezeri Erasto Ngowi

Investigation: Muhammad Shahid, Saadullah Khatak, and Ebenezeri Erasto Ngowi

Methodology: Muhammad Shahid, Saadullah Khatak, and Ebenezeri Erasto Ngowi

Resources: Salma S. Mahmoud, Hao-Jie Chen, Lei Qian, Yangzhe Qin, Dong-Dong Wu, Chun Yang Zhang, and Xin-Ying Ji

Software: Salma S. Mahmoud, Hao-Jie Chen, Lei Qian, and Yangzhe Qin

Supervision: Dong-Dong Wu, Chun Yang Zhang, and Xin-Ying Ji

Visualization: Salma S. Mahmoud, Hao-Jie Chen, Lei Qian, and Yangzhe Qin

Writing – original draft: Nazeer Hussain Khan and Wenkang Wang

Writing – review and editing: Tao Li, Muhammad Zubair, and Shazrul Fazry

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ORIGINAL RESEARCH ARTICLE

Fluorescence imaging-guided photothermal therapy of asymmetric water-soluble pentamethine cyanine for colorectal cancer

Kun Tang^{1†}, Shuangshuang Jia^{1†}, Yaxin Zou^{1†}, Jiaheng Dong¹, Fangyan Liu², Kunli Cui¹, Xiao Shi³, and Lei Zhang^{1*}

¹Henan International Joint Laboratory for Nuclear Protein Regulation, School of Basic Medical Sciences, Henan University, Kaifeng 475004, P. R. China

²Department of Academician Shengshou Hu Workstation, Fuwai Central China Cardiovascular Hospital, Zhengzhou 450046, P. R. China

³Department of Pharmacy, The First Affiliated Hospital of Henan University, Henan University, Kaifeng 475004, P. R. China

Abstract

The combination of advanced optical technology and tumor treatment has developed into a selectively targeted tumor therapy strategy. Near-infrared (NIR) cyanine derivative has recently received increasing attention in photothermal therapy (PTT) because of its excellent biocompatibility, non-immunogenicity, high fluorescence and photothermal conversion efficiency, photostability, and tumor-homing. In this study, an asymmetric and water-soluble pentamethine cyanine was designed and synthesized to investigate the photosensitive activity and to explore its potential applications as a NIR photosensitizer for the diagnosis and therapy of colorectal cancer (CRC). The results of fluorescence imaging analysis showed that CY5-664 preferentially accumulated in the tumor as time elapsed, and the maximum fluorescence intensity was obtained at 24 h. Cell viability and animal xenograft model experiments showed that CY5-664 significantly inhibited tumor growth *in vivo* with 0.8 W/cm² of 660 nm red laser pointer for 2 min, and *in vitro* with 0.5 W/cm² of 660 nm red laser pointer for 2 min. *In vitro* and *in vivo* photothermal conversion experiments showed that CY5-664 exhibited an anti-CRC activity mainly through the PTT effect. Therefore, this work provides experimental evidence that CY5-664 is a drug candidate for CRC treatment mainly through the PTT effect and an alternative treatment strategy to develop a pentamethine cyanine-based CRC theranostic photosensitizer for synergistic CRC targeting, imaging, and therapy.

[†]These authors contributed equally to this work

*Corresponding author:

Lei Zhang
(zhlei@henu.edu.cn)

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Keywords: Pentamethine cyanine; Photothermal therapy; Fluorescence imaging; Near-infrared; Colorectal cancer

1. Introduction

Colorectal cancer (CRC), a common clinical malignant tumor of the digestive tract, is seriously threatening human health due to the high incidence and mortality rates^[1]. The occurrence and development of CRC are usually related to diet changes and environmental factors^[2]. According to the statistics on cancer in 185 countries in 2018,

CRC incidence rate and mortality rate are among the top three in the world^[3]. Surgical treatment, radiotherapy, and chemotherapy are the principal clinical treatment methods of CRC^[4]. However, the common chemotherapeutic drugs, such as fluorouracil and oxaliplatin, have predictable hepatotoxicity^[5,6]. Therefore, it is urgent to develop an effective and low-toxicity drug for CRC treatment.

The combination of advanced optical technology and tumor treatment provides non-invasive *in vivo* imaging and treatment (optical diagnosis and therapy) employing non-ionizing radiation, which brings new understanding and technological innovation to biomedicine, and is expected to achieve precise treatment of tumors^[7,8]. Tumor phototherapy is a method of treating cancer with specific wavelength excitation photosensitizer, including photothermal therapy (PTT) and photodynamic therapy (PDT)^[9,10]. PTT, based on the theory concerning the absorbed light for heat conversion, has tremendous potential for efficacious cancer therapy, compared with traditional treatment modalities such as surgery, radiation therapy, and chemical therapy, due to the non-invasive therapeutic intervention and precise spatial-temporal selectivity^[11,12]. PDT is another new strategy for cancer therapy, accompanied by photosensitizers and laser activation. The laser energy is transferred to the surrounding oxygen by photosensitizer and highly active singlet oxygen is produced to kill cancer cells^[13]. However, the low oxygen content of the tumor microenvironment limits the extensive application of oxygen-dependent PDT^[14]. Compared with oxygen-dependent PDT, PTT is oxygen-independent and, therefore, may serve as a more promising cancer therapy approach. PTT photosensitizer effectively converts the laser energy into thermal energy through absorbing energy in the near-infrared (NIR) region and taking advantage of the sensitivity of cancer cells toward heat to induce hyperthermia and promote cancer cell necrosis and apoptosis^[15,16].

Tremendous efforts have been made to obtain high efficiency and low-toxicity PTT materials. Although a large number of polymeric and inorganic nanomaterials have been studied in the application of PTT, such as gold nanoparticles^[17], metal sulfide nanoparticles^[18], and carbon nanomaterials^[19], there are still tremendous challenges to clinically apply these PTT materials due to the complex synthetic process, the raised concerns regarding biocompatibility, and the unsolved biosafety problem^[16,20]. In contrast, NIR heptamethine cyanine fluorophore such as indocyanine green (ICG) recently received increasing attention in PTT because of excellent biocompatibility, non-immunogenicity, high fluorescence and photothermal conversion efficiency, photostability, tumor-homing, and commercial-scale production^[11,21,22]. Up to the present, ICG

was the only contrast medium authorized by the U.S. Food and Drug Administration. Therefore, the development of new photosensitizers based on the intrinsic multifunctional characteristics of their chemical structure, which do not rely on nanopolymer materials and do not need complex chemical connections, significantly improved the potential of clinical transformation and application, and promoted the prospects of tumor optical therapy.

In the present study, recognizing the potential of cyanine fluorophore, a unique water-soluble pentamethine cyanine-derivative, 1-(4-carboxybenzyl)-2,3,3-trimethylindoleninium-5-sulfonate bromide (CY5-664), was designed and synthesized to investigate the photosensitive activity and to explore its potential applications as NIR photosensitizer for CRC diagnosis and therapy. An excellent molar extinction coefficient, a relatively higher quantum yield, and good photostability are exhibited in CY5-664 aqueous solutions. CY5-664 inhibits cell viability and exhibits excellent PTT activity in two types of CRC cell lines in a dose-dependent manner under NIR laser irradiation. Moreover, it is retained preferentially in tumors and significantly inhibits tumor growth in the BALB/c nude mice xenografts bearing DLD-1 cells under NIR laser irradiation with negligible organ toxicity. Therefore, for the 1st time, we found the novel small molecular derivative of cyanine CY5-664 against CRC, which may be high efficiency and low-toxicity PTT material for image-guided CRC therapy in clinical application.

2. Materials and methods

2.1. Chemicals and reagents

CY5-664 was synthesized according to the predetermined synthetic route, and the synthetic routes were shown in Figure 1A. ¹H NMR spectra were recorded on a Bruker AMX-400 spectrometer (400 MHz; Bruker, U.S.A.). Mass spectral analysis was performed using an MALDI-TOF mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA, U.S.A.). Fluorescence measurement was performed using a Cary Eclipse spectrofluorimeter (Agilent Technologies, U.S.A.) with 5 nm excitation and emission slit widths. Absorption spectra were measured using a Jasco V-550 spectrophotometer (JASCO International Co., Japan). All reagents used for the experiments were of analytical grade. HSP70 and β -actin antibodies were purchased from Servicebio Co. Ltd. (Wuhan, China). MTT assay kit was purchased from Solarbio Co. Ltd. (Beijing, China). Singlet oxygen sensor green (SOSG) detection kit was purchased from Maokangbio Co. Ltd. (Shanghai, China).

2.2. Cell lines and cell culture

As previously described^[23], human CRC cell lines such as HCT-116 and SW480 were obtained from the cell culture

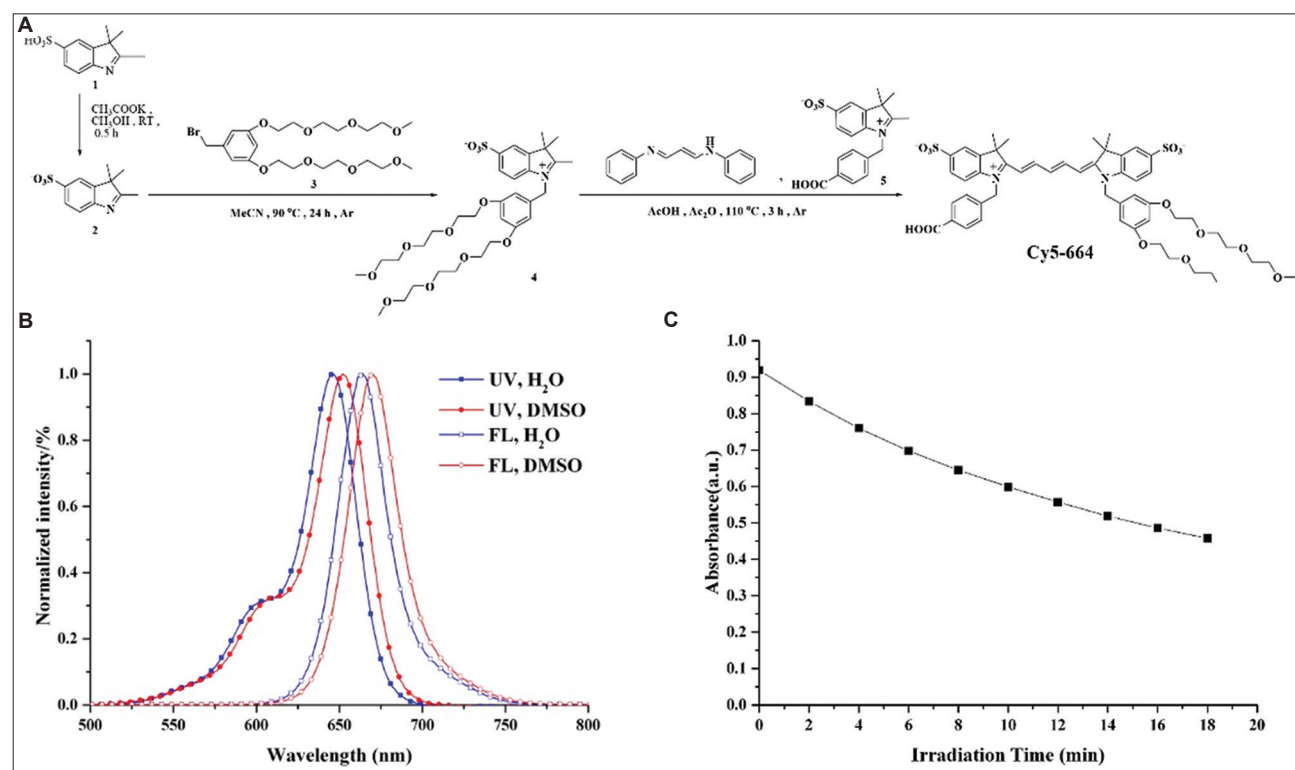


Figure 1. The synthesis scheme and spectral characteristics of CY5-664. (A) The synthesis scheme of CY5-664. (B) The spectral characteristics of CY5-664. (C) The photostability test of CY5-664 under laser irradiation (660 nm, 0.5 W/cm²) every 2 min.

collection committee of the Chinese Academy of Sciences library (Shanghai, China). The cells were cultured in RPMI 1640 (Gibco, 11875119) medium with 10% heat-inactivated fetal bovine serum (Gibco, 16000-044), 100 U/mL penicillin and 100 µg/mL streptomycin (Solarbio, P1400), and maintained in 5% CO₂ at 37°C.

2.3. Cell viability assay

As previously described^[24], HCT-116 and SW480 cells were seeded in 96-well plates at a density of 5×10^3 cells per well and cultured for 12 h. CY5-664 was added into culture plates according to the different predetermined concentrations, and the cells were cultured continuously for 2 h. Cells were irradiated for 2 min (660 nm, 0.5 W/cm²), and the cells were cultured continuously for 24 h. The cell viability was determined by the MTT method.

2.4. In vivo experiments

In vivo experiments of this study were approved by the Medical and Scientific Research Ethics Committee of Henan University School of Basic Medical Sciences. As previously described^[25], the mice used in this study were 5-week-old female BALB/c nude mice, which were purchased from Beijing Weitonglihua Experimental Animal Technical Co., Ltd (Beijing, China). Human

CRC cell HCT-116 was harvested and suspended in normal saline (NS), and was injected subcutaneously into nude mice at a concentration of 10^6 cells per nude mouse. The tumor volume was estimated according to the following formula: L (the long diameter) $\times$ W (the short diameter) $\times$ $W \times 1/2$. Nude mice were randomly divided into three groups: (1) NS + Laser, (2) CY5-664 treated only, and (3) CY5-664 + Laser, under laser irradiation (660 nm, 0.5 W/cm²) for 2 min, until the tumor size reached approximately 50 mm³. The CY5-664 treatment group mice were injected with CY5-664 at a concentration of 1 mg/kg, and NS control group mice were injected with an equal volume of NS through tail vein.

2.5. In vivo NIR fluorescence imaging

In vivo NIR fluorescence imaging was observed using an optical imaging system for small animals (IVIS lumina III, Perkinelmer, U.S.A.) at different predetermined time points after injection of CY5-664 through tail vein. Nude mice were dissected and pivotal organs and tissues, such as heart, liver, spleen, lung, kidney, and tumor, were obtained at 24 h after injection with CY5-664 through tail vein for further *ex vivo* biodistribution assessment. The excitation wavelength is 650 nm and the emission wavelength is 670 nm in NIR fluorescence imaging analysis.

2.6. Western blot analysis

The expression of HSP70 was detected by Western blotting according to the standard experimental schemes. As previously described^[26], human CRC cells HCT-116 and SW480 were collected and lysed with RIPA buffer in the presence of protease inhibitors. The protein was quantified and electrophoresed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After electrophoresis, the protein was transferred to the polyvinylidene fluoride (PVDF) membrane from the gel using the electrospinning method. The PVDF membrane was sealed with bovine serum albumin and coincubated with the primary antibody and the secondary antibody. Finally, the blot determination was detected using a chemiluminescence analyzer (Amerisham Biosciences, Boston, MA, U.S.A.).

2.7. Singlet oxygen detection analysis

The singlet oxygen generation from CY5-664 was determined by the fluorescent probe SOSG. In brief, 1.0×10^{-5} M Cy5-664 and 1.5×10^{-6} M SOSG were mixed in an aqueous solution containing 2% methanol and were irradiated with 0.5 W of 660 nm red laser pointer for 2 min at room temperature. The mixed solution was quickly transferred to the cuvette after laser irradiation and the fluorescence intensity was determined using a steady-state transient fluorescence spectrometer. The excitation wavelength is 494 nm and the emission peak at 530 nm was used to evaluate singlet oxygen generation.

2.8. In vitro assessment of photothermal effect

The photothermal effect of CY5-664 was determined using a precise thermal imaging instrument in a culture medium. In brief, different concentrations CY5-664 solutions were prepared according to the predetermined concentrations and were irradiated with 0.5 W of 660 nm red laser pointer for 2 min. The temperature of CY5-664 solutions was monitored by a precise thermal imaging instrument (Fotric 225S#L24, Shanghai, China) every 30 s.

2.9. In vivo photothermal analysis

BALB/c nude mice bearing human CRC cell HCT-116 were injected with NS or CY5-664 through tail vein. At 24 h after the injection of CY5-664, the tumor was irradiated by laser (660 nm, 0.5 W/cm²) for 2 min, and the local temperature of the tumor was monitored by a precise thermal imaging instrument (Fotric 225S#L24, Shanghai, China) every 30 s.

2.10. Hematoxylin & eosin (H&E) staining analysis

As previously described^[27,28], the tissues from the animal experiments were fixed in 4% paraformaldehyde fixative and embedded in paraffin. Formalin-fixed paraffin-embedded samples were sliced and stained with hematoxylin for 5 min

and eosin for 1 min. Morphological changes of the tumor tissues were observed with an electronic light microscope (Olympus, Tokyo, Japan).

2.11. Cell uptake assay

As previously described^[29], HCT-116 and SW480 cells were seeded in 24-well plates at a density of 5×10^3 cells per well and cultured for 24 h. Different concentrations of CY5-664 were added into culture plates, and the cells were cultured continuously for 2 h. The cells were washed 3 times with phosphate-buffered solution and detected using a fluorescence inverted microscope (DMI8, Leica, Germany).

2.12. PTT by CY5-664

HCT-116 and SW480 cells were seeded in 96-well plates at a density of 5×10^3 cells per well and cultured for 12 h. Different concentrations of CY5-664 were added to culture plates, and the cells were cultured continuously for 2 h. Cells were irradiated for 2 min (660 nm, 0.5 W/cm²) with or without ice incubation. The cell viability was determined by MTT method.

2.13. Statistical analysis

All statistical analyses were completed by SPSS16.0. The statistical difference between the treatment and control groups of IMCA was analyzed by Student's *t*-test.

3. Results and discussion

3.1. Synthesis and characterization of CY5-664

The synthesis routes are shown in Figure 1A. 2,3,3-trimethylindoleninium-5-sulfoacid (1), 1-bromomethyl-3,5-bis(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)benzene (3), and 1-(4-carboxybenzyl)-2,3,3-trimethylindoleninium-5-sulfonate bromide (5) were synthesized according to the literature^[30,31].

A mixture of 2,3,3-trimethylindoleninium-5-sulfoacid **1** (0.8 g, 3.34 mmol) and potassium acetate (0.36 g, 3.67 mmol) was stirred in 30 mL of methanol at room temperature for 0.5 h. After removal of methanol under vacuum, the resulting potassium salt **2** was heated with 1-bromomethyl-3,5-bis(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)benzene **3** (1.65 g, 3.33 mmol) in acetonitrile at 90°C for 24 h under argon. The mixture was cooled to room temperature and the solvent was evaporated. The pure product **4** (1.11 g) was obtained in 51% yield by the purification on silica gel column with CH₂Cl₂/CH₃OH (15:1, V/V) as eluent and the ¹H NMR spectrum is shown in Figure S1 (in Supplementary File).

The intermediate **4** (374.2 mg, 0.57 mmol) and malonaldehyde dianilide hydrochloride

(296.2 mg, 1.14 mmol) in a mixture of acetic anhydride (4 mL) and acetic acid (4 mL) were heated at 110°C for 1 h under argon. The intermediate **5** (638.8 mg, 1.71 mmol) was added, and then the mixture was heated at 110°C for another 3 h under argon. The blue solution was poured into 100 mL of ethyl acetate. The precipitate was filtered and washed with ethyl acetate. The pure product (**181.4 mg**) was obtained in 30% yield by the purification using reversed-phase (C18) chromatography with CH₃CN/CH₃OH (3:1, V/V) as eluent. The structural spectrum of CY5-664 is shown in Figures S2-S4.

Photofading experiments were carried out in the quartz cuvette (3.5 mL volume) containing a 5×10⁻⁶ M solution of the dye in ultrapure water, where the sample solution was irradiated with 0.5 W of 660 nm red laser pointer at room temperature. UV-vis spectra were recorded at 2-min intervals for 20 min. The irreversible bleaching of the dye at the absorption peak was monitored as a function of time. The highest absorption spectrum of CY5-664 was 646 nm and 652 nm, and the fluorescence peak was 664 nm and 670 nm in phosphate-buffered solution and dimethyl sulfoxide (DMSO) solution, respectively (Figure 1B). CY5-664 has a substantial absorption at 660 nm, which leads to photothermal conversion and fluorescence imaging. The absorption intensity decreases with the increase of laser irradiation (660 nm, 0.5 W/cm²) time, but the light absorption was not reduced significantly after 2 min of irradiation (Figure 1C). These results showed that CY5-664 remained stable after irradiation for 2 min.

3.2. Photoinduced cytotoxicity of CY5-664

Low toxicity under dark conditions and high toxicity under laser irradiation are the noteworthy characteristics of phototherapy^[32]. To evaluate the photoinduced cytotoxicity cancer specificity of CY5-664, cell viability was determined with or without CY5-664 treatment and 660 nm laser irradiation on HCT116 and SW480 cells. MTT was used as an indicator of cell viability. The results showed that different concentrations of CY5-664 did not significantly inhibit the viability of HCT116 and SW480 cells without laser irradiation, and Cy5-664 significantly inhibited the viability of the CRC cells in a dose-dependent manner. However, under laser irradiation, the viability of HCT116 and SW480 cells was significantly decreased with the increased concentration of CY5-664 (0–25 μM) (Figure 2). These results confirmed the photoinduced cytotoxicity of CY5-664 in a dose-dependent manner.

3.3. Imaging, phototherapy, and *in vivo* toxicity evaluation

To demonstrate the tumor-targeting properties and the tumor imaging capability of CY5-664 *in vivo*, BALB/c nude

mice bearing human CRC cell HCT-116 xenografts model were established as described previously^[23]. CY5-664 was injected into the nude mice bearing HCT-116 tumor xenografts at a dose of 1 mg/kg through the tail vein and the NIR fluorescent imaging was carried out from 30 min to 96 h after injection using a non-invasive imaging system^[33]. The results of fluorescence imaging analysis showed that CY5-664 preferentially accumulated in the tumor as time elapsed, and the maximum fluorescence intensity was obtained at 24 h (Figure 3A). To further confirm the tumor-targeted accumulation of the compounds, nude mice were dissected and pivotal organs and tissues, such as heart, liver, spleen, lung, kidney, and tumor, and were obtained at 24 h after injection with CY5-664 through tail vein^[34]. The obtained organs and tissues were analyzed by a fluorescence imaging system. The results of fluorescence imaging analysis showed that CY5-664 was confirmed with significant tumor preferential accumulation by *ex vivo* imaging of the dissected organs and tissues (Figure 3B).

To evaluate the antitumor activity of CY5-664 *in vivo*, HCT116 subcutaneous tumor xenograft models were established, and the tumor-bearing nude mice were randomly divided into three groups: (1) NS + Laser, (2) CY5-664 treated only, and (3) CY5-664 + Laser. HCT116 subcutaneous tumor xenograft models were established on both shoulders of each mouse in the CY5-664 treatment group. The tumor on one shoulder was irradiated by laser, while the tumor on the other shoulder was not irradiated by laser. The tumor in NS group mice was irradiated by laser. According to the results of NIR fluorescence imaging described above, tumors in laser irradiation group were irradiated with 660 nm laser for 2 min (0.8 W/cm²) at 24 h after injection. The results are shown in Figure 3C-E. Compared with the NS group, tumor growth was significantly retarded in the CY5-664 + Laser irradiation group. Similar results were further observed by means of tumor imaging and tumor weight analysis in the CY5-664 + Laser irradiation group. The antitumor effect of CY5-664 was further evaluated using H&E staining of tumor tissue sections. As shown in Figure 3F-H, all of the tumor cells in the NS-treated group with laser irradiation and CY5-664-treated group without laser irradiation displayed deeper hematoxylin staining, larger nuclei, complete morphology, and vigorous division. In contrast, the tumor cells in the CY5-664 + Laser irradiation group showed lighter hematoxylin staining, damaged nuclear integrity, and shrunk chromatin. These results showed that CY5-664 significantly inhibits tumor growth with laser irradiation *in vivo*.

To evaluate the toxic side effects of CY5-664 *in vivo*, the weight of nude mice was monitored in real time during the treatment. As displayed in Figure 3I, compared with the

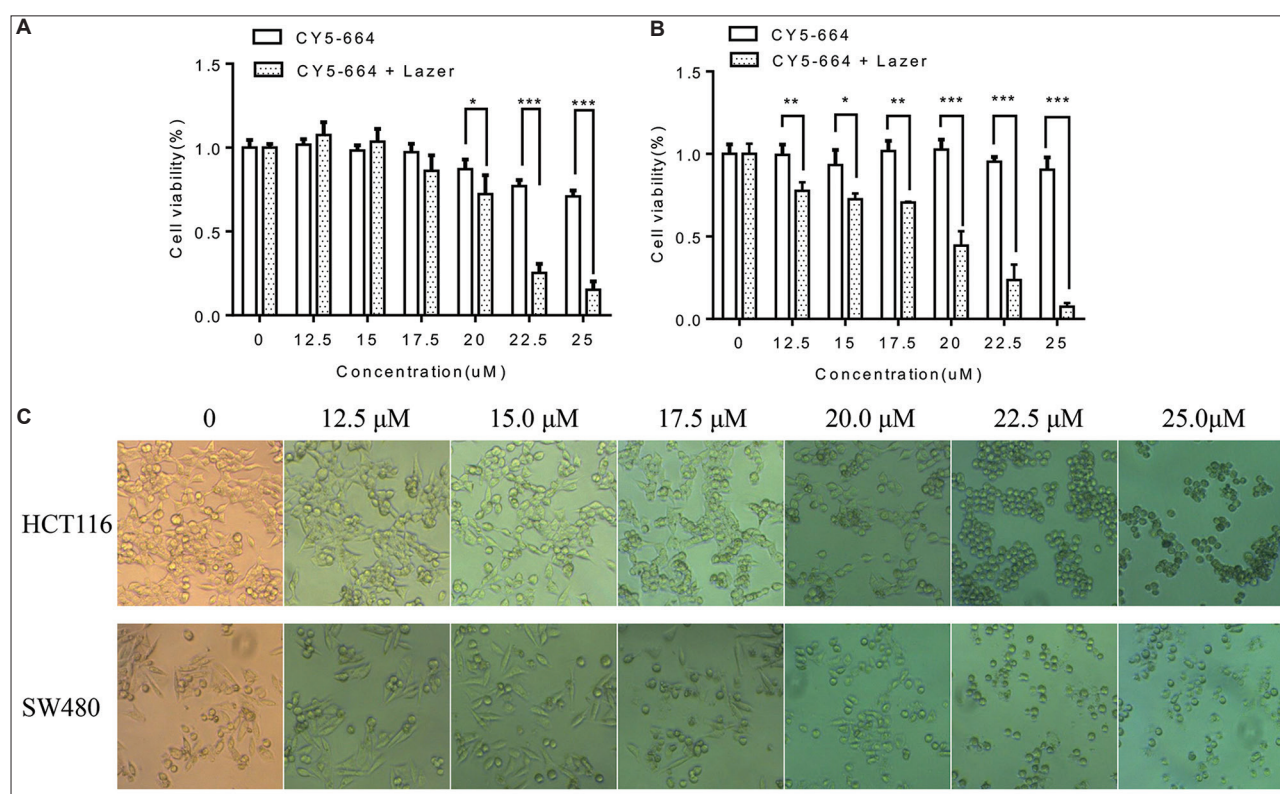


Figure 2. Photoinduced cytotoxicity of CY5-664. (A) CY5-664 induced significant cytotoxicity under laser irradiation on HCT-116 cells. (B) CY5-664 induced significant cytotoxicity under laser irradiation on SW480 cells. (C) Morphological pictures of cells treated with different concentrations of CY5-664 under laser irradiation on HCT-116 and SW480 cells. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

control group, there was no significant difference in body weight during the whole observation period. Liver and kidney functions were further determined by biochemical tests of alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (Cre), and blood urea nitrogen (BUN). As shown in Figure 3J-M, there was no significant difference in these indicators between the CY5-664 + Laser irradiation group and the control group. To further evaluate the effects of CY5-664 on the main organs, we carried out the organ index and H&E staining analysis of the main organ sections, such as the heart, liver, spleen, lung, and kidney. The results showed that no significant changes in organ index and abnormal histomorphology were observed (Figure 3N and S). Overall, the above results indicated that tumor-targeted CY5-664 and imaging-guided phototherapy were highly biocompatible and low in toxic side effect.

3.4. The mechanism of CY5-664 phototherapy

Photodependent cancer therapy, including PTT and PDT, has emerged as a local and non-invasive therapeutic method for cancer therapy in recent years^[35]. For PTT, light energy is absorbed and converted into hyperthermia by a photosensitizer, achieving cancer therapeutic activity^[36].

The photothermal activities of CY5-664 were evaluated at three levels: Medium, intracellular, and nude mice. Excitedly, the temperature of the media containing different concentrations of CY5-664 increased rapidly, and the highest temperature reached 68.9°C in the media containing 50 μM CY5-664 under laser irradiation (660 nm, 0.5 W/cm², 2 min) (Figure 4A). To further evaluate the effect of photothermal treatment of CY5-664 on cell viability, cell viability was determined by the MTT method with or without ice incubation for different concentrations of CY5-664 under laser irradiation (660 nm, 0.5 W/cm², 2 min). The results showed that ice significantly reversed the inhibition of CY5-664 on cell viability under laser irradiation (Figure 4B and C). Heat shock protein 70 (Hsp70) is a member of the most ancient and universal defense systems, which can be synthesized under heat stress to achieve the purpose of cell protection^[37]. To study the photothermal effect of CY5-664, the expression of HSP70 was determined using Western blotting in HCT116 and SW480 cell lines. The results showed that the expression of HSP70 had been significantly upregulated (Figure 4D). Based on the phototherapeutic efficacy of CY5-664 *in vitro*, *in vivo* phototherapeutic activity of CY5-664 was initially evaluated in HCT-116 subcutaneous

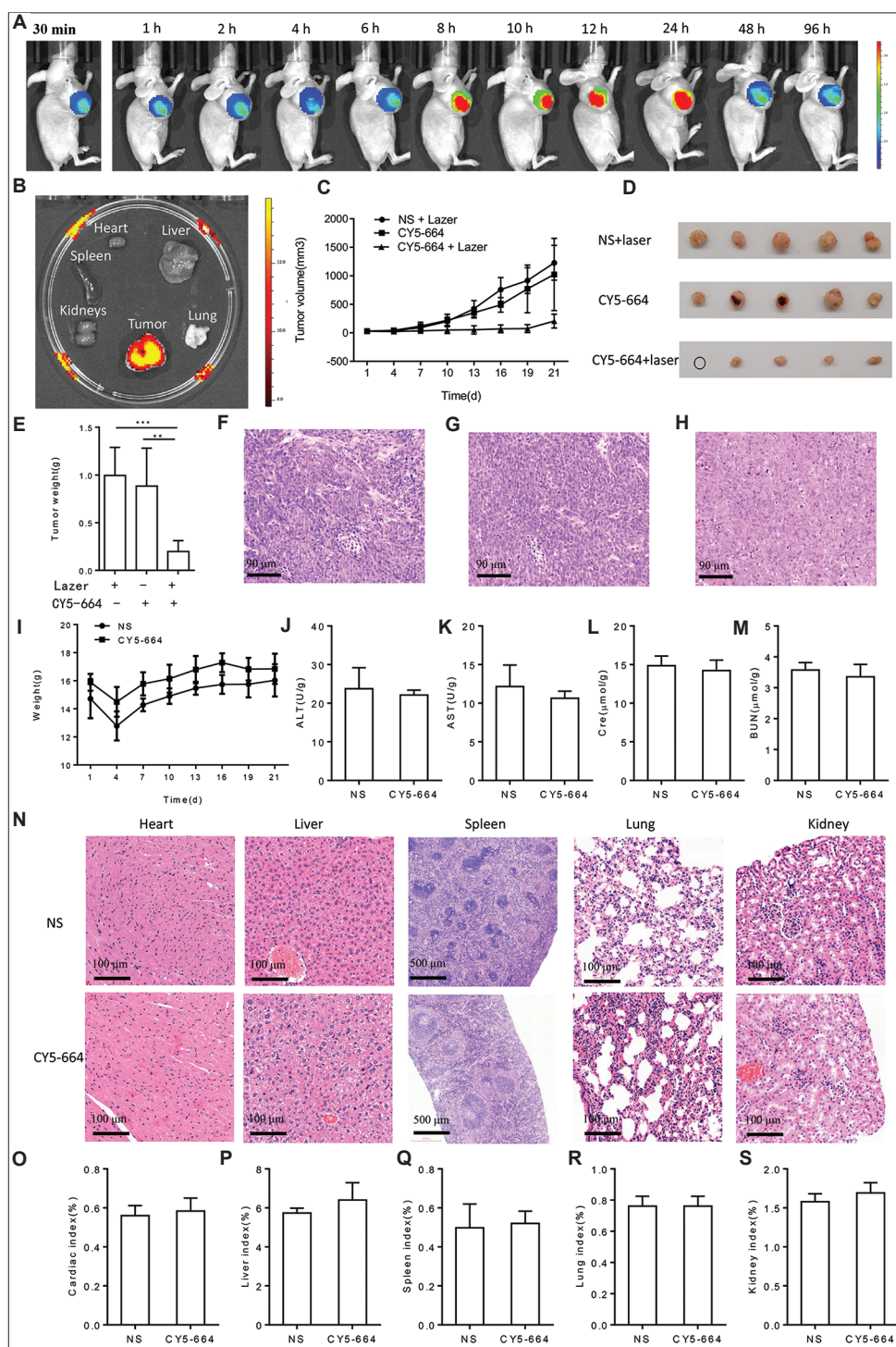


Figure 3. The biodistribution, tumor inhibition and side effects of CY5-664 *in vivo*. (A) The fluorescence imaging at different times after injection of CY5-664. (B) The fluorescence imaging of main organs and tumor at 24 h after injection. (C) The change of tumor volume during the observation period. (D) Photos of the tumor after treatment. (E) Tumor quality after treatment. (F–H) The morphological characteristics of tumor tissue, NS + Laser (F), CY5-664 (G), and CY5-664 + Laser (H). (I) The change of body weight during the observation period. (J) The change of ALT in the liver. (K) The change of AST in the liver. (L) The change of Cre in the kidney. (M) The change of BUN in the kidney. (N) The morphological characteristics of main organs with or without CY5-664 treatment. (O–S) The change of main organs. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

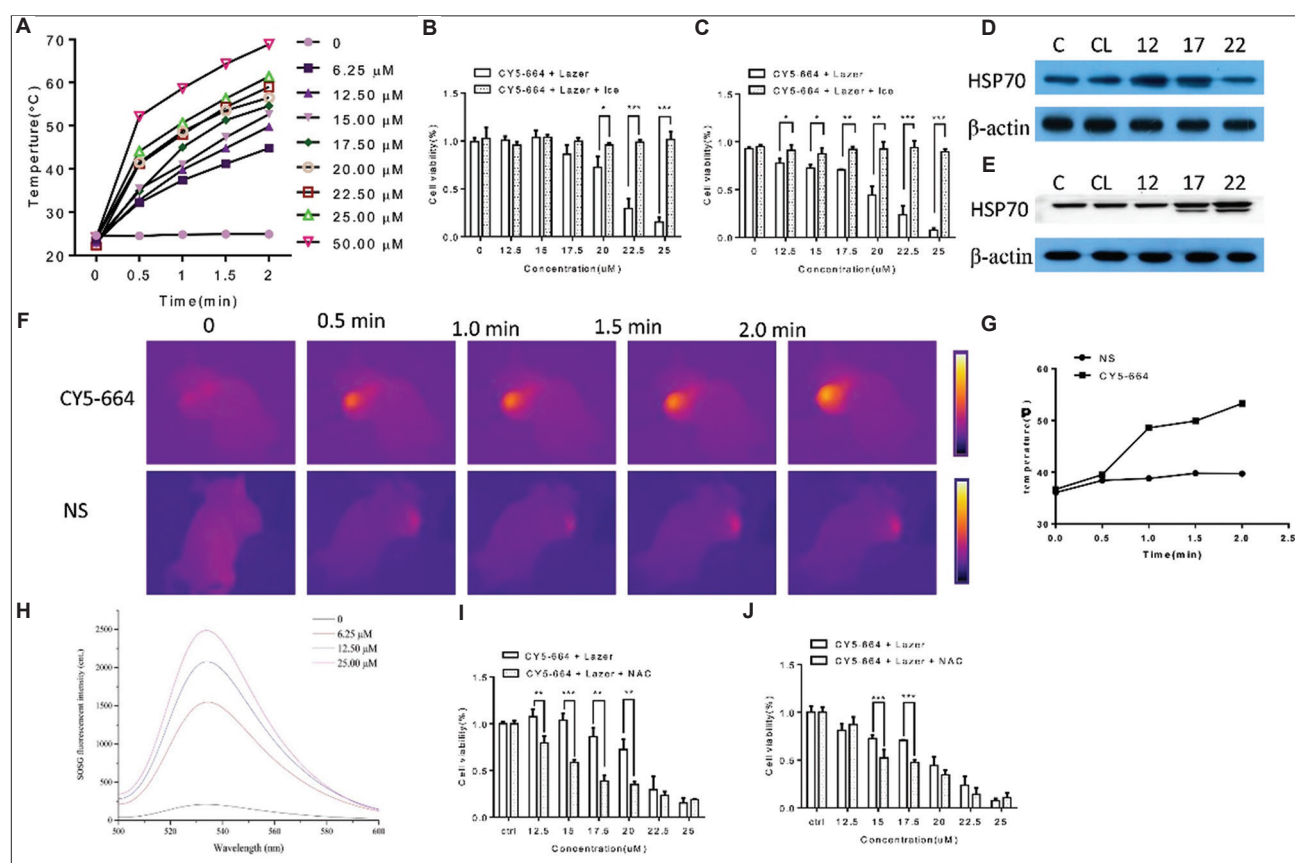


Figure 4. Evaluation of the photosensitive effect of CY5-664 *in vitro* and *in vivo*. (A) The temperature change of CY5-664 solution under laser irradiation (660 nm, 0.5 W/cm², 2 min). (B) The cell viability of HCT-116 cells with or without ice incubation for different concentrations of CY5-664 under laser irradiation (660 nm, 0.5 W/cm², 2 min). (C) The cell viability of SW480 cells with or without ice incubation for different concentrations of CY5-664 under laser irradiation (660 nm, 0.5 W/cm², 2 min). (D–E) The HSP70 expression in HCT-116 (D) and SW480 (E) cells under laser irradiation (660 nm, 0.5 W/cm², 2 min); C: control, CL: control + laser. (F) The infrared ray thermal images during the 2-min laser irradiation (660 nm, 0.5 W/cm², 2 min). (G) The temperature curve during the 2-min laser irradiation (660 nm, 0.5 W/cm², 2 min). (H) The singlet oxygen generation of CY5-664 solution under laser irradiation (660 nm, 0.5 W/cm², 2 min). (I) The cell viability of HCT-116 cells with or without NAC for different concentrations of CY5-664 under laser irradiation (660 nm, 0.5 W/cm², 2 min). (J) The cell viability of SW480 cells with or without NAC for different concentrations of CY5-664 under laser irradiation (660 nm, 0.5 W/cm², 2 min). **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

tumor-bearing xenograft models under the guidance of photothermal imaging. CY5-664 was injected into the tumor-bearing nude mice through the tail vein at 24 h before the laser and then the tumor was irradiated for 2 min using a 660 nm laser (0.8 W/cm²). Meanwhile, the surface temperature of the tumor is monitored every 30 s using a thermal camera. As shown in Figure 4F and G, the surface temperature of the tumor rose rapidly and reached 60°C at 2 min after laser irradiation in CY5-664 treatment group mice. However, the surface temperature of the tumor only slightly increased to 43°C in NS groups mice. In summary, the antitumor activities of CY5-664 were closely related to the strong photothermal activities.

For PDT, light energy is absorbed and converted into reactive oxygen species by a photosensitizer, achieving cancer therapeutic activity^[38–40]. Based on the photothermal

property of CY5-664 *in vitro* and *in vivo*, the photodynamic activities of CY5-664 were further determined by measuring the singlet oxygen production with SOSG. The results showed that singlet oxygen production was significantly induced by CY5-664 under laser irradiation (Figure 4H). To further evaluate the intracellular photodynamic property of CY5-664, cell viability was determined by the MTT method with or without antioxidant N-acetyl-L-cysteine (NAC) for different concentrations of CY5-664 under laser irradiation (660 nm, 0.5 W/cm², 2 min). However, as shown in Figure 4I and J, the inhibition of HCT-116 and SW480 cell viability induced by CY5-664 under laser irradiation was not rescued by NAC. The cellular uptake of CY5-664 was further determined by fluorescence microscope analysis at 2 h post-treatment of CY5-664. As shown in Figure S5, CY5-664 was not uptaken by HCT116 and SW480 cells. These results showed that CY5-664 did

not induce colorectal cell death through the photodynamic effect.

4. Conclusions

In summary, an asymmetric, water-soluble pentamethine cyanine derivative CY5-664 with integrated imaging, targeting, and therapeutic properties has been found in this work. Significant anti-CRC activity of CY5-664 was observed by the multifunctional photosensitizer through cell viability and animal xenograft model experiments. This multifunctional photosensitizer CY5-664 may have great application prospects as a safe and effective reagent for treating CRC because of the following advantages. First, CY5-664 preferentially accumulates in the tumor as time elapsed, and the maximum fluorescence intensity was observed at 24 h. Second, CY5-664 has good water solubility and can be dissolved in a cell culture medium or NS without DMSO. Third, CY5-664 can be imaged *in vivo*, which is conducive to its application in imaging-guided photosensitive therapy. Finally, CY5-664 exhibits significant PTT effects for CRC therapy without significant side effects. In brief, this study not only found a multifunctional photosensitizer CY5-664, but also provided an alternative treatment strategy to develop pentamethine cyanine-based CRC theranostic photosensitizer for synergistic CRC targeting, imaging, and therapy.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Conceptualization: Kun Tang, Shuangshuang Jia, Yaxin Zou, Jiaheng Dong, and Lei Zhang

Formal analysis: Kun Tang

Investigation: Shuangshuang Jia, Yaxin Zou, Jiaheng Dong, Fangyan Liu, Kunli Cui, and Xiao Shi

Methodology: Kun Tang, Shuangshuang Jia, Yaxin Zou, Jiaheng Dong, and Lei Zhang

Writing – original draft: Kun Tang

Writing – review & editing: Lei Zhang

All the authors read and agreed to publish the version of this study.

Availability of data and materials

Samples of the compounds are available from the authors.

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REVIEW ARTICLE

Gene-modified T cell therapy for cancer: Current challenges and potential solutions

Xinfeng Chen^{1,2†}, Shasha Liu^{1,2†}, Zhen Zhang^{1,2}, and Yi Zhang^{1,2,3,4*}

¹Biotherapy Center, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan, China

²Cancer Center, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan, China

³School of Life Sciences, Zhengzhou University, Zhengzhou 450052, Henan, China

⁴Engineering Key Laboratory for Cell Therapy of Henan Province, Zhengzhou, 450052, Henan, China

Abstract

Immunotherapy has achieved significant breakthroughs in patients with hematological diseases and various cancers. The adoptive transfer of T cells, especially gene-modified T cells such as chimeric antigen receptor T (CAR-T) cells and T cell receptor-engineered T (TCR-T) cells, is developing rapidly. Since 2017, eight CAR-T cell products have been approved for the treatment of hematological diseases, such as refractory or relapsed acute B lymphoblastic leukemia, specific subtypes of B cell lymphoma, and multiple myeloma. The first TCR-T cell product, Kimmtrak, was approved for the treatment of unresectable or metastatic uveal melanoma in March 2022. However, there are still many problems, including side effects, relapse, high demand for individualization, and expensive cost in hematological diseases, tumor heterogeneity, antigen escape, poor immune cell infiltration ability, immunosuppressive microenvironment, and low response in solid tumors. With the in-depth exploration of tumor immunology and the development of genetic engineering technology, many novel strategies for improving the anti-tumor effect and safety of gene-modified T cell immunotherapy have been attempted. This paper presents a systematic review of the literature on CAR-T cell and TCR-T cell therapy, focusing on applications, clinical trials, problems, and potential solutions.

Keywords: Immunotherapy; Chimeric antigen receptor T cell; T cell receptor-engineered T cell; Tumor microenvironment; Solid tumor

[†]These authors contributed equally to this work.

*Corresponding author:

Yi Zhang
(yizhang@zzu.edu.cn)

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1. Introduction

In recent years, chimeric antigen receptor T (CAR-T) cell therapy has become one of the most promising therapeutic methods in cancer immunotherapy because of the remarkably effective and durable immune response it produces in hematological malignancies^[1,2]. CD19 and B cell maturation antigen (BCMA) CAR-T cell products have been approved for clinical applications worldwide^[3]. This has promoted further large-scale clinical trials of CAR-T cells in other malignant diseases. However, CAR-T cell therapy is afflicted with several problems, such as toxicity, resistance or relapse, and

hyperthermia in solid tumors. Optimization of CAR structures to reduce side effects and improve their efficacy in solid tumors has been widely studied to solve these problems.

Another gene-engineered T cell therapeutic pattern is T cell receptor-engineered T (TCR-T) cell therapy, which exhibits unique advantages in the treatment of solid tumors^[4]. TCR-T cells evoke a cytotoxic response and induce tumor regression *in vivo*^[5]. To date, only one TCR-T cell product (Kimmtrak) has been approved for the treatment of unresectable or metastatic uveal melanoma^[6]. More clinical trials of tumor-specific/associated antigen- or neoantigen- modified TCR-T cells in solid tumors are underway^[7]. While various challenges exist in the application of adoptive T cell therapy, basic research and clinical trials have been conducted to mitigate these limitations. As the technology develops, the accumulation of clinical trial data will support the approval of more engineered T cell products and benefit more patients with tumors. In this review, we discuss the current, challenges, and recent advances in the use of gene-engineered T-cells in cancer immunotherapy. Furthermore, we highlight potential strategies to conquer the disadvantages of extending adoptive T cell therapy.

2. The concept and generation of CAR-T cell therapy

CAR-T cells are prepared using gene-editing technology (i.e. viral or non-viral vector transfection) to endow common T cells with a single-chain variable fragment (ScFv) containing recognized tumor antigens and signals of T cell activation, producing specific recognition, and cytotoxic functions independent of major histocompatibility complex expression^[8]. Specifically, CAR-T cells rely on ScFv in the CAR structure, which recognizes tumor antigens and promotes T cell activation and expansion^[9]. Activated CAR-T cells secrete cytokines, such as interleukin (IL)-2 and interferon (IFN)- γ , which can induce apoptosis of target cells. In 1987, Kuwana *et al.* developed the first CAR prototype by fusing the V region of immunoglobulin with the constant region of the TCR^[10]. The first-generation CAR construct contained the ScFv and CD3 signaling regions, which endowed T cells with transient activation and cytotoxicity, but insufficient anti-tumor efficacy^[11]. In subsequent studies and clinical trials, second-generation CAR-T cells were developed, which integrated costimulatory signaling molecules CD28 or CD137 into the intracellular signaling domain improving the cytotoxicity and existence of CAR-T cells^[12,13]. With second-generation CAR-T cells targeting CD19 produces a high objective response rate (ORR) in relapsed/refractory (R/R) acute B cell lymphocytic leukemia (B-ALL), specific

types of B cell lymphoma and chronic B cell lymphocytic leukemia, and several autologous CAR-T cell products targeting CD19 have been approved, providing novel therapeutic strategies for R/R B cell malignant diseases^[14]. Meanwhile, the products of CAR-T cells targeting BCMA have been approved by the U.S. Food and Drug Administration (FDA) in the treatment of R/R multiple myeloma^[3]. However, plenty of clinical trials have been conducted on second-generation CAR-T cells in the treatment of solid tumors without any breakthroughs^[15]. Scientists have extensively explored solutions to the current issues with second-generation CAR-T cells, such as reducing toxicity by altering CAR structure or expressing a “suicide gene”^[16], and improving the therapeutic effect with dual-target CAR-T cells^[17,18]. Expression of two costimulatory molecules enhances the amplification capacity and anti-tumor effect of CAR-T cells, which are regarded as third-generation CAR-T cells^[15]. Chemokine receptors, cytokines, or transcription factor-modified CAR-T cells are named fourth-generation CAR-T cells^[19]. Meanwhile, dual- or three-target modified CAR-T cells for preventing antigen escape, or other gene-editing strategies for improving the efficacy are defined as next-generation CAR-T cells. The clinical application and basic study of CAR-T cell therapy in hematological malignancies and solid tumors are described in detail below.

3. CAR-T cell therapy in hematological malignancies

Many clinical trials of differentially targeted CAR-T cells in various hematological malignancies are currently underway, including CD19, CD20, CD7, CD30, CD38, BCMA, CD22, CD70, CD79b, and dual targets. It was reported that 80 – 94% of R/R B-ALL patients who received CD19-targeted CAR-T cell infusion achieved complete remission (CR)^[20]. Moreover, 80% of diffuse B-cell lymphoma patients had an objective response, and nearly 40% had a long progression-free survival^[21]. The result of ZUMA-2, a large clinical trial of CD19 CAR-T cell product TECARTUS, showed that 67% of patients with R/R mantle cell lymphoma achieved a CR^[22]. ZUMA-5 indicated that R/R indolent non-Hodgkin's lymphomatous (NHL) patients receiving axicabtagene ciloleucel therapy had a high overall response (92%) and CR (74%)^[14]. More clinical trials have been conducted to evaluate the potential of CD19 CAR-T cells as a first- or second-line therapeutic method for large B-cell lymphoma^[1,2]. When 41 R/R Hodgkin's lymphoma patients received a CD30 CAR-T cell infusion, the total OR and CR rates were 72% and 59%, respectively^[23]. CAR-T cells (idecabtagene vicleucel) targeting BCMA have a high response rate in R/R multiple myeloma and have been approved by the U.S. FDA. In a

study of 33 R/R multiple myeloma patients who were treated by bb2121 CAR-T cells, with a total OR rate of 85%, 15 (45%) of patients achieved CR, with a median disease-free progression survival of 11.8 months^[24]. At present, approved CAR-T cell products are all indicated for R/R hematological malignancies targeting CD19 or BCMA.

4. Issues of CAR-T cell therapy in hematological malignancies

Although CAR-T cells produced a high ORR in hematological malignancies, it has certain limitations, including related toxicity in the treatment process, high demand for individualization, long production cycle, expensive production cost, no response and relapse after remission in some patients. Cytokine release syndrome (CRS) and on target, off-tumor toxicity are specific side reactions of CAR-T cell therapy^[25]. In addition to toxicity, resistance occurs during CAR-T cell therapy. With an increase in the number of patients receiving CD19 CAR-T cells and long-term follow-up data, 30 – 50% of B-ALL patients receiving CD19 CAR-T cells who achieved a CR would relapse within 1 year^[26]. Moreover, 10 – 20% of patients still show no response after treatment with CD19 CAR-T cells^[27]. Relapse in patients with multiple myeloma receiving BCMA CAR-T cell infusion is frequent as well^[28]. Thus, resistance after CAR-T cell therapy is a major problem in the field of cancer immunotherapy, and understanding the mechanism of resistance and non-responsiveness is critical for enhancing the anti-tumor effect of CAR-T cell therapies. Existing studies have confirmed that functional defects in CAR-T cells, the tumor cell mutations, and tumor microenvironment are implicated in the primary or secondary resistance of B cell malignant disease to CAR-T cells^[29]. The following sections describe these problems and their existing and potential solutions.

5. Strategies to overcome issues in CAR-T cells for hematological malignancies

5.1. Toxic reactions of CAR-T cell therapy

CRS is considered a specific side effect related to CAR-T cell infusion, which mainly manifests as fever, hypotension, hypoxia, organ dysfunction, hemocytopenia, coagulation dysfunction, and hemophagocytic lymphohistiocytosis^[30]. CRS is an inflammatory syndrome caused by a variety of cytokines produced by activated CAR-T cells and other immune cells. If CAR-T cells recognized target cells, they secrete large amounts of the cytokines IFN- γ and tumor necrosis factor alpha (TNF- α), which activate monocytes and macrophages to produce large amounts of pro-inflammatory cytokines, predominantly IL-6^[25,31]. IL-6 can activate other immune cells through classical

signaling pathways, thus further activating T cells in a “positive feedback” manner. It can act on other cells such as endothelial cells to cause systemic inflammatory reactions, mainly involving monocyte Chemoattractant Protein (MCP)-1, IL-8, and IL-6, increased secretion of endothelial permeability factors such as vascular hemophilia factor, and reduced levels of endothelial stabilizer angiotensin 1, which causes loss of vascular integrity, hemodynamic instability, capillary leakage syndrome, and coagulation dysfunction^[32]. Moreover, nitric oxide produced by macrophage-derived nitric oxide synthase can further enhance hemodynamic instability and vasodilation; therefore, macrophage activation plays a key role in CRS development^[33]. The risk factors of CD19 CAR-T cell therapy induced-CRS mainly include high tumor burden, high-doses of CAR-T cells, expansion of CAR-T cell *in vivo*, thrombocytopenia and endothelial cell activation, the status of myelosuppression after pretreatment, and costimulatory molecules of CD28^[15,34,35]. At present, scientists have found that IL-1, catecholamine activation, and pyroptosis of target cells can activate macrophages and participate in the pathogenesis of CRS related to CAR-T cell therapy^[36-38]. Thus, a dissection of the mechanisms of CRS production caused by CAR-T cell therapy will be beneficial for exploring therapeutic strategies to control CRS and elevate the safety during CAR-T cell therapy. The cytokines closely related to CRS mainly include IL-6, IL-1, IL-10, TNF- α , and IFN- γ ^[20]. The IL-6 receptor blocker tocilizumab has been approved for the treatment of CRS produced by CAR-T cell therapy. Glucocorticoids (dexamethasone or methylprednisolone) can be used to treat suboptimal CRS after treatment with tocilizumab injection^[25]. In addition, plasma purification (hemofiltration or plasmapheresis) is an effective therapeutic method for handling CAR-T cell infused-related cytokine storm^[39,40]. JAK and IL-1 pathway inhibitors have also been shown to control CRS^[36,41]. Activation of the catecholamine system participates in the CRS process, and whether β -receptor blockers can control CRS or reduce grades should be further explored^[37]. One of the means to improve safety is altering the CAR structure expressing suicide gene^[42], which can selectively remove CAR-T cells, but increase the risk of disease recurrence. Altering the affinities of the ScFv structure of CAR did not affect its efficacy, but the associated side effects were reduced^[43]. Twenty patients with B-cell lymphoma who were treated with Hu19-CD828Z T cells had lower cytokine levels compared to those treated with FMC63-28Z T cells, and the severe neurotoxicity incidence rate was only 5%^[44]. In short, the control of CRS should follow the principles of prediction, close monitoring, timely intervention, and prevention (Figure 1).

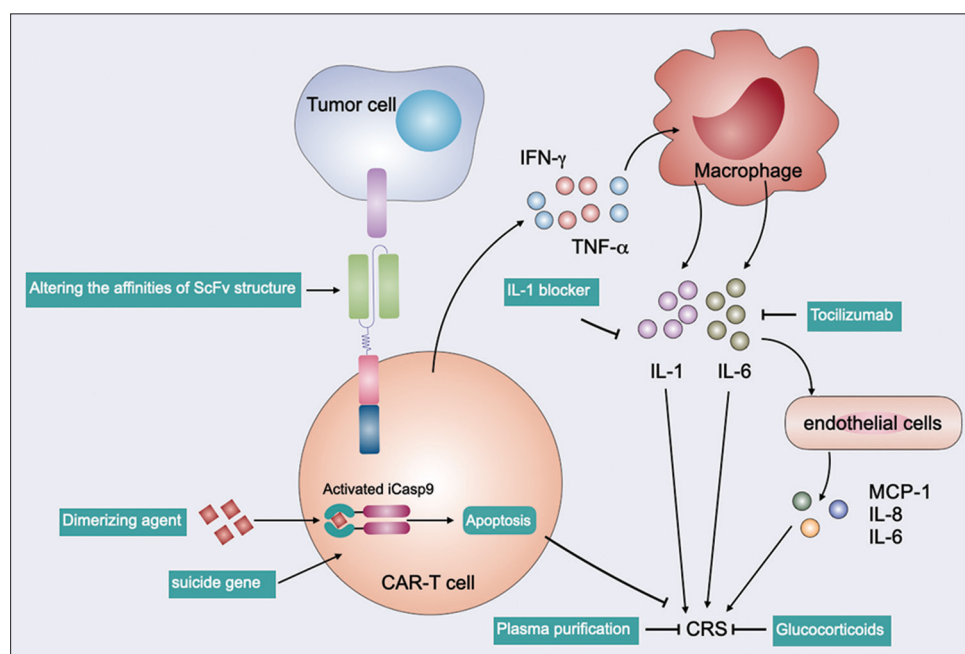


Figure 1. The molecular mechanism and therapeutic intervention of CRS produced by CAR-T cells. On recognition of target cells, CAR-T cells produce IFN- γ and TNF- α , which activate monocytes/macrophages to produce large amounts of pro-inflammatory cytokines, predominantly consisting of IL-6 and IL-1. IL-6 could activate other immune cells through classical signaling pathways, thus further activating T cells and setting a “positive feedback loop.” In addition, they can also act on other cells such as endothelial cells to cause systemic inflammatory reactions, mainly by releasing MCP-1, IL-8, and IL-6 to induce CRS. Therapeutics targeting the IL-6 receptor (tocilizumab) and IL-1 receptor can mitigate CRS. Reforming the CAR structure-expressed suicide gene or treatment with a dimerizing agent to activate iCasp9 to induce apoptosis can enhance the safety of CAR-T cells. Glucocorticoids and plasma purification (hemofiltration or plasmapheresis) are effective therapeutic methods for managing CAR-T cell therapy-related CRS. Altering the affinities of ScFv structure in CAR reduces associated side effects.

5.2. Potential strategies to improve the efficacy of CAR-T cell therapy

5.2.1. Improving the persistence of CAR-T cells

The existence of CAR-T cells is associated with a durative anti-tumor ability *in vivo*^[45]. T cell exhaustion is a crucial factor participating in the resistance to CAR-T cell therapy^[46]. In the process of target cell activation, some immunosuppressive molecules will upregulate their expression, such as programmed cell death protein 1 (PD-1), and the expression of the ligand of immunosuppressive molecules (i.e. PD-L1) is increased on the surface of tumor cells or other immune cells. Activation of the axis of PD-1/PD-L1 pathway negatively regulates the anti-tumor function of CAR-T cells^[47]. CAR-T cells combined with PD-1 blockers were able to produce synergistic anti-tumor immune response^[48]. CRISPR/Cas9-edited PD-1 negative CAR-T cells exhibited enhanced anti-tumor ability *in vitro* and *in vivo*^[49]. Some patients with B-cell lymphoma could benefit from the treatment with pembrolizumab after the failure of CD19 CAR-T cell therapy^[50]. It was reported that Bruton's tyrosine kinase (BTK) inhibitors could improve the immune response of CD19 CAR-T cells *in vitro* and *in vivo*^[51]. Patients with B-cell lymphoma resistant to the

first CD19 CAR-T cell infusion could benefit from BTK inhibitor salvage therapy and re-treatment with the same CAR-T cell product^[52]. Exploring the mechanism of CAR-T cell exhaustion could guide novel strategies for improving anti-tumor effect. DNMT3A-mediated T cell exhaustion has been reported to be regulated by epigenetics^[53]. When exposed to a low dose of decitabine, CAR-T cells exhibit enhanced anti-tumor immune responses, cytokine expression and proliferation^[54]. Overexpression of c-Jun regulated by Ap-1 could inhibit the exhaustion of CAR-T cells^[55]. Basic leucine zipper ATF-like transcription factor (BATF) was confirmed to be participating in the regulation of T cell function through inhibiting the exhaustion-associated marker expression and enhancing the production of effector cytokines^[56]. Combining CAR-T cells with an immunopotentiator or altered CAR structure to prevent exhaustion will enhance their anti-tumor ability (Figure 2).

5.2.2. Antigen escape: Dual/three-target modified CAR-T cells

Depending on the analyzed data of the molecular characteristics of relapsed patients infused with CD19 CAR-T cells, single-target CAR-T cells are prone to

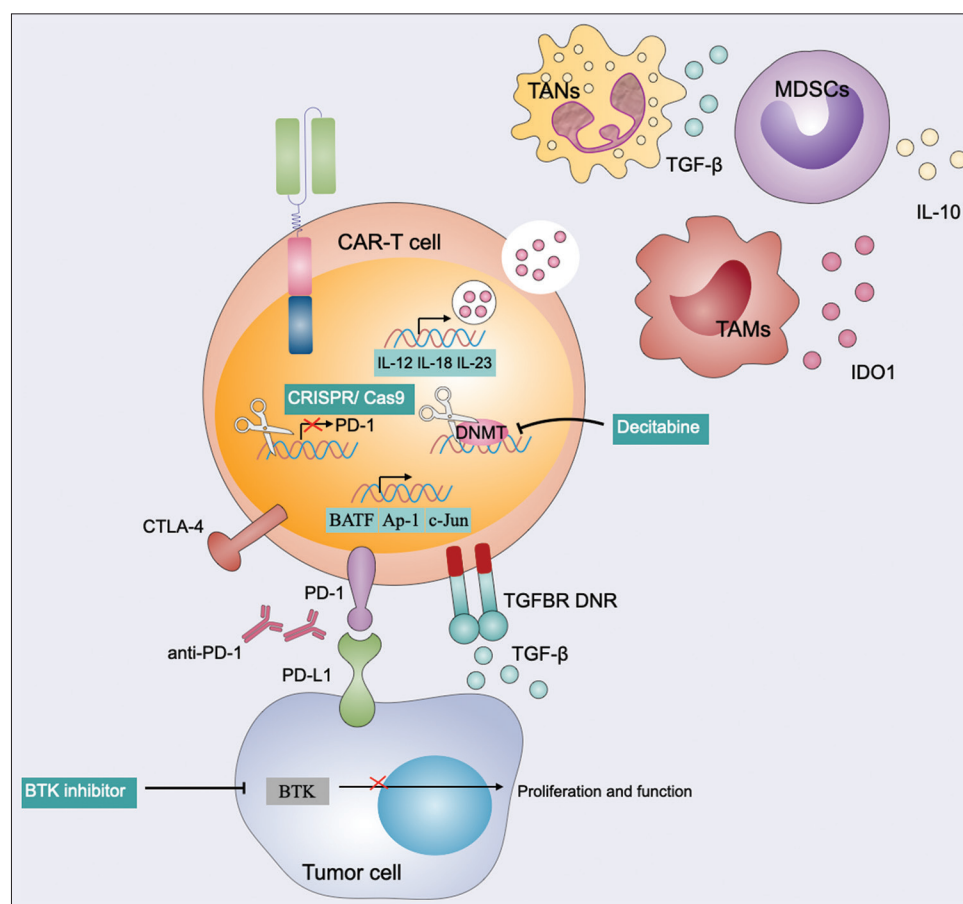


Figure 2. Strategies of promoting the persistence of CAR-T cells. Using immune checkpoint inhibitors, BTK inhibitors or a low dose of decitabine could improve the persistence of CAR-T cells. The existence of CAR-T cells *in vivo* can be improved by CRISPR/Cas9-edited PD-1 or TGF- β R2, overexpression of c-Jun, Ap-1 and BATF, or arming CAR-T cells through overexpressing pro-inflammatory cytokines, for instance, IL-12, IL-18, or IL-23.

immune escape, and 50% of patients experienced target-antigen negative relapse. It has been confirmed that dual- or three-target CAR-T cells could boost the anti-tumor effects of CAR-T cells and reduce immune escape *in vitro* and *in vivo*^[57]. Moreover, the CR rate of patients with B-cell lymphoma treated with CD19 CAR-T cells is lower than that with R/R B-ALL. Increasing the CR rate of CAR-T cell treatment in B-cell lymphoma is the focus of current research trend. Han *et al.* reported that CD19/CD20 dual-target CAR-T cells have been displayed to increase the CR rate of patients with B-cell lymphoma. In a phase I/IIa clinical trial, 33 patients with R/R B cell lymphoma were recruited, 28 of whom received pretreatment and infused with CAR-T cells, the total response and CR rates was 79% and 71%, respectively, and 14% of patients experienced grade 3 CRS^[57]. In a trial containing 16 patients with R/R B-NHL treated by CD19/CD22 dual-target CAR-T cells, the overall response rate was 87.5%, the CR rate was 62.5%, and the 2-year overall survival and disease progression-free survival rates were 77.3% and 40.2%, respectively^[58]. Similarly, treatment with

CD19/CD22 CAR-T cells similarly showed a higher CR rate in patients with R/R B-ALL^[59]. In one patient with primary B-cell lymphoma of the central nervous system, CD19/CD70 CAR-T cells induced a CR acquisition, and no significant side effects were observed^[60]. CD19/BCMA CAR-T cells had enhanced anti-tumor effect in multiple myeloma^[61]. About 87% of R/R multiple myeloma patients who received CD38/BCMA CAR-T cells attained a clinical response, with 52% achieving a stringent CR at a median 9.0 months follow-up^[62]. Therefore, the use of dual- or multi-target CAR-T cells for the treatment of hematological malignant disease will improve the CR rate in lymphoma and reduce the recurrence rate of B-ALL. This provides a theoretical and practical basis for the exploration of this treatment in solid tumors^[63] (Figure 3).

5.3. Reducing costs with universal CAR-T (U-CAR-T) cells

At present, CAR-T cell products that have been approved for clinical application are individualized therapies with

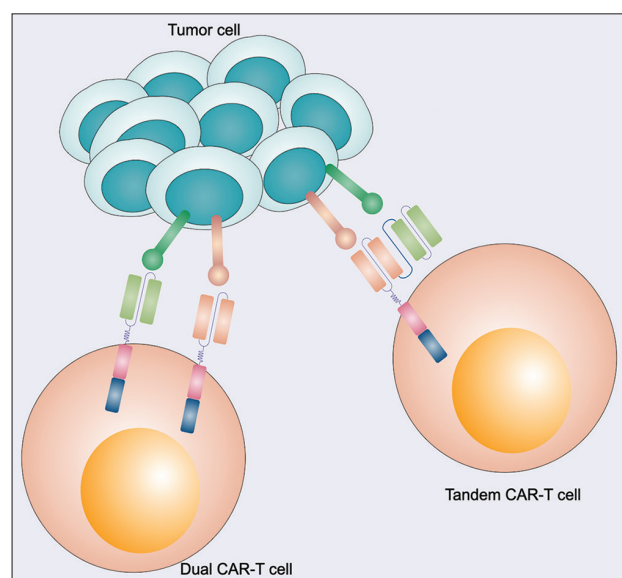


Figure 3. Reducing antigen immune escape with dual- or multiple- target CAR-T cells. Multiple CAR and tandem CAR structures are designed to improve the ability of CAR-T cells to recognize different antigens on tumor cell surface for preventing antigen immune escape.

many limitations. First, due to their specific matching as well as long production preparation and quality control period, patients with rapid disease progression may miss the treatment opportunity for CAR-T cell infusion. Second, in infants, severe patients, and some patients with immune system damage, their own T cells cannot be used for CAR-T cell preparation or will undergo culture failure, and they will not be able to receive autologous CAR-T cell infusions. Moreover, the consistency of individual products is difficult to guarantee and the production cost is high, thereby limiting the large-scale production of CAR-T cells and their wide application. Therefore, exploring U-CAR-T cells has become a focus in the field of immunotherapy^[64,65]. TALEN and CRISPR/Cas9 are the two most commonly used gene-editing methods. The main targets of U-CAR-T cell clinical trials include CD19, BCMA, CD7, CD22, CD123, CS1, and mesothelin^[66]. Allogeneic CD19 CAR-T cell by silencing T-cell receptor α constant (TRAC) was first reported in 2012^[67]. TALEN-edited CD19 CAR-T cells of CD52⁺/TCR⁺ killed target cells with high specificity^[68]. CRISPR/Cas9 editing of allogeneic CAR-T cells with multiple genes has been reported, and studies have shown that the product of pure CAR expression is safe and efficient, and can direct CAR to the TRAC loci of TCR in T cells^[69]. Simultaneous CD52 knockdown in TCR gene-edited CAR-T cells, combined with CD52 monoclonal antibody, inhibited the immune cell activity in the host and enhanced the expansion of allogeneic CD19 CAR-T cells, and produced an anti-leukemic effect^[70].

Twenty-one patients with R/R B-ALL received U-CAR-T cell infusion, and CR or non-hematologic CR was achieved in 14 patients within 28 days. CRS occurred in 91% of these patients, three patients had Grade 3 – 4 CRS, two patients experienced Grade 1 skin rejection, and eight patients manifested Grade 1 – 2 neurotoxicity. It has been reported that CD19/CD22 dual-target U-CAR-T cells created by CRISPR/Cas9-edited TRAC and CD52 produced a CR rate of 83.3% without dose-limiting toxicity, graft-versus-host disease, neurotoxicity, or other adverse events associated with genome editing in six R/R B-ALL patients^[69]. Clinical trials of allogeneic CAR-T cells in other malignancies are in progress.

6. CAR-T cell therapy in solid tumors

CAR-T cell therapy was first intended for use in treating solid tumors, but due to the specificity of the target, the complexity of the tumor microenvironment and tumor heterogeneity, T cell recruitment, and other factors caused poor efficacy in solid tumors^[71]. With the development of efficient gene-editing technology, an endless stream of CAR-T therapies has emerged. The selection of CAR-T cell-targeted antigen has important effects on both therapeutic efficacy and safety. Unlike hematological tumors, the therapeutic targets in solid tumors are mostly over-expressed antigens. Thus, those tumor-associated antigens (TAAs) with relatively specific expression and higher individual positivity rates may represent optimal targets for CAR-T cell therapy. At present, the main targets of relevant clinical trials in solid tumors are GPC3, mesothelin, EGFR, PSPA, CEA, and GD2. Although the development of CAR-T cell therapy is rapid, there are still many limitations and challenges in solid tumors. These major challenges include tumor heterogeneity and antigen escape, insufficient immune cell infiltration, tumor immunosuppressive microenvironment, T cell exhaustion, and dysfunction. The clinical trial status of different targets in different tumors is detailed below (Table 1). Thirteen patients with hepatocellular carcinoma infused with a median dose of 19.9×10^8 GPC3 CAR-T cells, eight patients experienced Grade 1 – 2 CRS without Grade 3 – 4 neurotoxicity, two patients achieved partial responses (PR), and one patient maintained a stable disease (SD) and was alive after 44.2 months^[72]. Six patients with metastatic pancreatic ductal adenocarcinoma received autologous mesothelin-specific CAR-T cell infusion; none of them experienced CRS and neurotoxicity, two patients achieved SD with progression-free survival times of 3.8 and 5.4 months^[73]. Sixteen patients with metastatic pancreatic carcinoma were treated with 1 – 3 cycles of the epidermal growth factor receptor (EGFR)-CAR-T cells within half a year, 4 of 14 evaluated patients achieved PR for 2

Table 1. Major published trials of engineered T cell therapy in solid tumors.

Type	Target	Clinical trial no.	Disease	Pre-chemotherapy	Cell dose	Number of patients	CR	PR	SD	PD	ORR
CAR-T	GPC3	NCT02395250 NCT03146234	HCC	Cyclophosphamide fludarabine	$1 - 3 \times 10^5/\text{kg}$	13	/	2	1	/	2
CAR-T	mesothelin	/	PDAC	/	$1 - 3 \times 10^8/\text{m}^2$	6	/	/	2	4	/
		NCT02414269	MPM	Cyclophosphamide		25	2	/	8	/	2
CAR-T	EGFR	NCT01869166	PC	Nab-paclitaxel Cyclophosphamide	$0.3 - 60 \text{ M/kg}$	16	/	4	8	/	4
CAR-T	CEA	NCT02349724	CRC	Cyclophosphamide Fludarabine	$1 \times 10^5 - 1 \times 10^8/\text{kg}$	10	/	/	2	7	/
CAR-T	Her2	NCT00902044	Sarcoma	/	$1 \times 10^4 - 1 \times 10^8/\text{m}^2$	19	/	/	4	1	/
		NCT01935843	BTC/PC	Nab-paclitaxel Cyclophosphamide	$2.1 \times 10^6/\text{kg}$	11	/	1	5	5	1
CAR-T	Claudin18.2	NCT03874897	GC	/	$2.5 - 5 \times 10^8 \text{ cells}$	28	/	16	5	7	16
TCR-T	NY-ESO-1	NCT03399448	MM	Cyclophosphamide Fludarabine	/	3	/	/	2	/	2
TCR-T	MAGE-4	NCT04044768	SSC	Cyclophosphamide Fludarabine	/	37	2	11	15	/	13
TCR-T	E7	NCT02858310	HPV-associated carcinomas	Cyclophosphamide Fludarabine	$1 \times 10^{11} \text{ cells}$	12	/	6	4	/	6

BTC: Biliary tract cancers; CR: Complete remission; CRC: Colorectal cancer; GC: Gastric cancer; HCC: Hepatocellular carcinoma; HPV: Human papillomavirus; MM: Multiple myeloma; MPM: Malignant pleural mesothelioma; ORR: Objective response rate; PC: Pancreatic cancer; PD: Progressive disease; PDAC: Pancreatic ductal adenocarcinoma; PR: Partial response; SD: Stable disease; SSC: Synovial sarcoma.

– 4 months, and eight patients had SD for 2 – 4 months. Downregulation of EGFR expression on surface of tumor cells has been found in patients with SD^[74]. Twenty-eight patients with gastric cancer received CLDN18.2-targeted CAR T cells, the ORR and disease control rates were 57.1% and 75.0%, respectively, and the percent of overall survival at six months was 81.2%^[75]. Two of ten patients with colorectal cancer achieved SD after CEA-CAR-T cell infusion without any severe adverse events^[76]. In general, the safety and efficacy of different antigens targeted by second-generation CAR-T cells have been widely evaluated in various cancers. Low incidences of CRS and low responsiveness were observed in solid tumors, indicating that the process of CAR-T cell therapy has been promising but tortuous.

6.1. Tumor heterogeneity and antigen escape

Antigen recognition is a prerequisite for an anti-tumor immune response. Antigen-specific TCR-T or CAR-T cells exert potent anti-tumor effects by targeting TAA. An important reason for the success of immunotherapy in hematological tumors is the existence of clear, uniformly expressed tumor antigens and controllable toxicity. However, due to the heterogeneous expression of solid tumor antigens in terms of intensity and distribution, the TAA currently used for solid tumor treatment is highly

expressed in tumor tissues. Downregulation of antigens participates in the resistance to CAR-T cell therapy^[77]. To overcome antigen escape, dual- or multiple-target CAR-T cells have been explored in several cancer types^[63].

6.2. CAR-T cell trafficking into tumor site

$\gamma\delta$ T cells have a natural tumor microenvironment chemotaxis, and GD2- $\gamma\delta$ CAR-T cells have a strong anti-tumor immune response, suggesting that $\gamma\delta$ CAR-T cells might be more dominant in solid tumors^[78]. The chemokine-chemokine receptor axis is a key factor influencing the accumulation of T cells in the tumor fraction, and the combination of chemokines released by tumor cells and chemokine receptors on T cells promotes T cell infiltration into the tumor site. CAR-T cells targeting mesothelin expressing CCR2b can enhance migration to tumor sites and their anti-tumor capacity^[79]. Moreover, GD2-specific CAR-T cells expressing IL-7 and CCR2b have a stronger capacity for tumor infiltration and anti-tumor effects in the neuroblastoma model^[80]. Studies have shown that the ligand of CXCR2 is highly expressed in hepatocellular carcinoma tissues and cell lines, while infiltrating T cells express lower levels of CXCR2, and CAR-T cells overexpressing CXCR2 produce enhanced anti-tumor activity by improving the infiltration of CAR-T cells^[81]. Overexpression of IL-7 and CCL19 (7×10^9

CAR-T cells) could increase the infiltration and existence of CAR-T cells^[82]. GPC3 and mesothelin CAR-T cells expressing IL-7 and CCL19 produce efficient anti-tumor response in patients with hepatoma and pancreatic cancer, respectively^[83]. In these studies, chemokine receptor- or cytokine-modified CAR-T cells showed significant tumor localization and anti-tumor activity *in vivo* (Figure 4).

6.3. Immunosuppressive microenvironment

In the tumor microenvironment, immunosuppressor cells such as myeloid-derived suppressor cells, regulatory T cells (Tregs), tumor-associated macrophages (TAMs), and various cytokines contribute to tumor progression dependent on promoting tumor invasion and metastasis and inhibiting the T cell immune response^[84]. Overcoming the immunosuppressive tumor microenvironment would elevate the anti-tumor capacity of adoptively transferred T cells in solid tumors^[85-87]. Arming CAR-T cells through expressing pro-inflammatory cytokines such as IL-12, IL-18, or IL-23 can remodel the tumor microenvironment and improve anti-tumor capability^[88-90]. Transforming growth factor beta (TGF- β) is a vital factor for tumor initiation and progression, which negatively regulates anti-tumor immunity of T cells. It has been reported that TGF- β could promote T cell exhaustion through up-regulating PD-1 expression in cytotoxic CD8⁺ T cells^[91,92]. CRISPR/Cas9-edited TGF- β R2 enhances the anti-tumor capability of CAR-T cells by reducing the transformation

of Tregs and T cell exhaustion^[93]. Thirteen patients with metastatic castration-resistant prostate cancer received prostate specific antigen-directed CAR-T cells armored with a dominant-negative TGF- β R; among the patients, five patients experienced grade $\geq$ r CRS, and 4 patients experienced a reduction in prostate-specific antigen level^[94]. The density of TAMs is negatively correlated with the prognosis in various types of solid tumors. Regulating the polarization of macrophages would reshape the tumor microenvironment. For example, folate receptor β (FR) β is highly expressed on the surface of macrophages, and FR β +TAM is related to poor outcome in pancreatic cancer. FR β -CAR-T cells could delay tumor progression through eliminating TAM, increasing pro-inflammatory monocytes and promoting endogenous tumor-specific CD8⁺ T cell infiltration^[95].

7. TCR-T cell therapy

Tumor-specific lysis of T cells is mediated by specific TCR-recognizing peptides and HLA complex^[96]. TCR-engineered T cells refers to the genetic editing of T cells *in vitro* by transducing TCR gene sequences (α and β chains) to T cells, resulting in enhanced ability of T cells to specifically recognize tumor antigens on the tumor cell surface and effectively induce specific lysis of target tumor cells. The TCR expressed by the exogenous gene binds CD3 molecules in a non-covalent bond, forming a TCR-CD3 complex expressed on the cell membrane, which further recognizes the HLA-antigen peptide complex. Unlike CAR-T cells, TCR-T cells can recognize HLA peptides derived from intracellular proteins and kill target cells in HLA-restricted dependent manner^[96-98]. The expression of cancer/testis antigens is silent in normal tissues, except in reproductive system, but is significantly increased in tumor tissues, including melanoma-associated antigen (MAGEA)-3, MAGEA-4, MART-1, gp100, and New York esophageal squamous cell carcinoma (NY-ESO-1), which are considered attractive targets for TCR-T cell therapy^[99]. Approximately 37% of published clinical trials are related to TCR-T cell therapy targeting NY-ESO-1^[100]. In 2011, the first clinical trial of autologous NY-ESO-1-specific TCR-T cells on advanced malignant melanoma and synovial cell sarcoma was conducted; 4 of 6 synovial cell sarcoma patients and 5 of 11 melanoma patients achieved ORR, and with a CR after one year was observed in two melanoma patients^[5]. MAGE-A4 TCR-T cells produced significant anti-tumor ability in patients with esophageal cancer^[101]. However, off-target toxic effects were observed in several patients who received MAGE-A3-specific TCR-T cell infusion^[102]. Fatal neurotoxicity and lethal cardiac toxicity occurred due to cross-reactivity of MAGE-A3-specific TCRs in the brain and myocardium with a

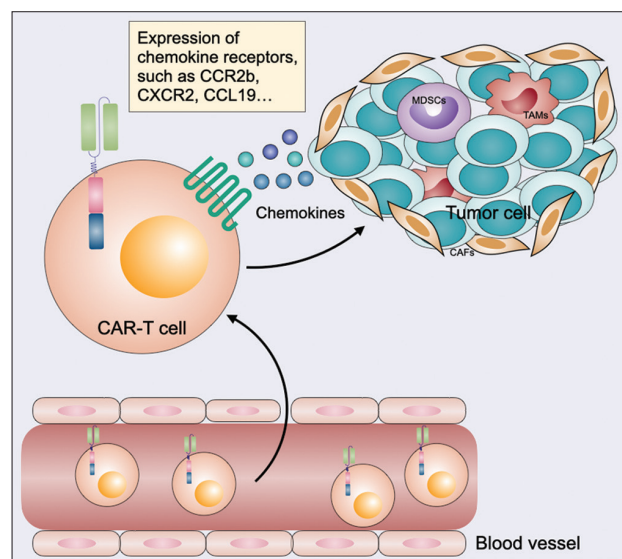


Figure 4. Improving CAR-T cells infiltrating into the tumor site. In tumor microenvironment, large quantities of chemokines are secreted, overexpression of the chemokine receptors, such as CXCR2 and CCR2b, can promote the infiltration of T cells into the tumor site. Overexpression of CCL19 in CAR-T cells enhances the infiltration of T cells and dendritic cells (DCs) into tumor site.

high affinity^[102,103]. Although TCR-T cell immunotherapy has shown some clinical efficacy in most treated patients, some obstacles remain to be overcome to realize the real promise of TCR-T cells therapy. Some nonresponsive patients lack *in vivo* persistence of T cell infusion in clinical trials, suggesting that TCR-T cells require additional support to improve their persistence *in vivo*^[104]. T cell infiltration into the tumor is not observed in some patients with late recurrence, and the infused TCR-T cells confront with an unfavorable immunosuppressive tumor microenvironment. In general, the application of TCR-T cells is still a challenge in many areas, including targeted immunotoxicity caused by normal tissues, low transient efficiency of TCR in engineered T cells, exhaustion and dysfunction of T cells, tumor immune escape and the lack of effective tumor-specific antigens in most patients with tumors^[105]. Therefore, overcoming these challenges is key to achieving greater clinical success in the future.

8. Improving the safety and efficacy of TCR-T cell therapy

The persistence and expansion of antigen specific T cells *in vivo* are related to the response to TCR-T cell therapy^[98,106]. The function of tumor antigen-specific T cells expressing exhaustion marker in the tumor is impaired^[107]. Exhaustion limits the anti-tumor immune response of T cells, and combination of immune checkpoint inhibitors with TCR-T cells could produce synergistic anti-tumor effect. Like in CAR-T cells, IL-7 and CCL19 were constructed into TCR-T cells to generate potent and durable anti-tumor immunity when synergizing with PD-1 blockade therapy^[108]. Co-expressing PD1-41BB-modified PRAME-specific TCR-T cells produced strong anti-tumor reactivity in an animal model^[109]. After identifying TCR sequences that specifically targeting neoantigens, TCR-T cells were reconstructed so that they are equipped with the potential anti-tumor ability^[110].

9. Outlooks

The remarkable success of adoptive T cell therapy for hematological tumors (B-ALL, B-cell lymphoma, and MM) makes CAR-T cells a promising therapeutic method for tumors, and promotes the rapid development of gene-modified T cell therapy. However, there are significant differences in anti-tumor mechanisms between CAR-T cells and TCR-T cells, such as structure, target, affinity, costimulatory molecule, and HLA limitation. They have the same issues, including toxicity, poor efficacy, poor infiltration, and immunosuppressive microenvironment in the treatment of solid tumors. The optimization of CAR or TCR structure will further improve its efficacy and safety, and facilitate the development of solid tumor treatment.

We believe the accumulation of findings from basic research and clinical translation will hasten and support the approval of more gene-engineered T cell products for clinical applications.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Supervision: Yi Zhang

Writing – original draft: Xinfeng Chen, Shasha Liu

Writing – review & editing: Xinfeng Chen, Shasha Liu, Zhen Zhang

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PERSPECTIVE ARTICLE

Constructing human genetic disease database in Bangladesh

Fariya Akter¹, Yusha Araf², Saeed Anwar³, Mohammad Jakir Hosen^{2*}, and Chunfu Zheng^{4,5*}¹Biotechnology Program, Department of Mathematics and Natural Sciences, School of Data and Sciences, BRAC University, Dhaka, Bangladesh²Department of Genetic Engineering and Biotechnology, School of Life Sciences, Shahjalal University of Science and Technology, Sylhet, Bangladesh³Department of Medical Genetics, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Alberta, Canada⁴Department of Immunology, School of Basic Medical Sciences, Fujian Medical University, Fuzhou, China⁵Department of Microbiology, Immunology and Infectious Diseases, University of Calgary, Calgary, Alberta, Canada

Abstract

In the advent of technological revolution, genetic interaction has become a crucial aspect in the understanding of any diseases. Information on individual genetic variation is now used in translational genomics to implement precise diagnosis and personalized medicine. Finding causal genes for genetic diseases or exploring interaction of genes in diseases with genetic predisposition is the first step to getting insights into such diseases. The human genome project made a paradigm shift in thinking, especially in the developed countries affected by non-communicable diseases. Some cutting-edge technologies, including gene therapy and genome editing, hold the promise in better diagnosis and treatment of common to rare genetic diseases. Scientific communities are trying hard to accumulate all the genetic disease information from publicly available platforms. A genetic disease database of a country serves as a depository. Many developed and a few developing countries have already developed genetic disease databases, which could benefit early diagnosis and proper patient management. Unfortunately, Bangladesh is lagging behind in this aspect. It is imperative to develop genetic disease database in Bangladesh because of its large population of patients with genetic disease. In this review, we discuss the reasons for constructing a genetic disease database and how this database can help to fight against challenges arising from the genetic diseases in Bangladesh.

***Corresponding authors:**Mohammad Jakir Hosen
(Jakir-gen@sust.edu)
Chunfu Zheng
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1. Introduction

Genetic disease or disorder is caused by the mutation(s) in one or more genes or anomalies in the chromosome^[1]. The precise diagnosis, control, and management of a genetic disease depend on the identification of causal variants in the genome^[2]. The breakthrough discovery of the human genome project allows scientists to understand the disease better. With

the advent of technological revolution and gold standard Sanger sequencing, massively parallel DNA sequencing has revolutionized the identification of novel human genetic variations. As a result, 20,442 coding genes and more than 15,000 monogenic diseases have been discovered^[3]. Multi-omics and translational research also make precision medicine and gene therapy possible for many diseases^[4].

On the other hand, the incidence of non-communicable diseases, including cardiovascular diseases, diabetes, and cancers with genetic predisposition, has increased alarmingly worldwide. Genetic variants in chronic disease provide a better understanding for screening, diagnosis, and precise early treatment^[5]. Pharmacogenomics using genome-based tools allow for administration of precise drug doses to eliminate toxicity and improve efficacy^[6]. Thus, genetic information has become a key component for efficiently diagnosing and managing any diseases.

Genetic diseases are emerging at an alarming rate in both developed and developing countries. According to the World Health Organization, about 2 – 3% of people suffered from genetic diseases globally by birth, and over 70% of these disorders are preventable^[7]. It has been reported that approximately 65% of people have some health problems resulting from genetic mutations, one in 50 people is affected by a single-gene mutation, and around one in 263 people is affected by chromosomal abnormalities^[8]. Remarkably, the prevalence of genetic diseases varies between populations. Thus, the population- or country-specific mutation spectrum of genetic diseases is important as a guide for controlling and properly managing genetic diseases. Unfortunately, genetic research remains neglected in low-income and lower-middle-income countries, including Bangladesh. Most developed countries and a few low-income and lower-middle-income countries have

their own genetic disease databases, but a genetic disease database is currently unavailable in Bangladesh.

In this paper, we discuss the reasons for constructing a genetic disease database and review the status quo of genetic disease research in Bangladesh, which can aid in the management of genetic diseases.

2. Status of genetic disease research in Bangladesh

Bangladesh is a highly dense country with a population of 166 million people, of which 90% are Muslim, and consanguineous marriage is very prevalent in the country^[9]. Inhabitants of Bangladesh are suffering from many infectious and non-infectious diseases. Among non-infectious diseases, genetic diseases and congenital malformations are quite common. Since there is neither systematic genetic testing nor well-developed expertise for counseling nationwide, most genetic diseases in Bangladesh remain undiagnosed and have become a significant health burden^[10]. Through extrapolating from the worldwide prevalence of genetic diseases, it is presumed that there are many patients with genetic diseases in Bangladesh. A comprehensive literature review revealed that more than 60 case reports on genetic diseases have been published, summarized in Table 1. Only nine of the available case reports demonstrated the utilization of genetic testing (possibly done from abroad) to confirm the presence of a specific disorder. Whole-genome sequencing has been done for only four Bangladeshi individuals, revealing over 11,500 variants responsible for different diseases and 17 genetic diseases^[11]. Another whole-exome sequencing study discovered the presence of pathogenic variants in other genes associated with an extremely rare genetic diseases in five unrelated patients^[12].

Table 1. A detailed summary of published case reports on genetic diseases in Bangladesh.

Genetic diseases	Inheritance mode	Diagnosis procedure	Publishing year of the first case report	Reference
Achondroplasia	Autosomal dominant	(1) Medical history collection (2) Physical examination	2020	[13]
Attention-deficit hyperactivity disorder (ADHD)	Autosomal dominant	(1) Interview (2) Identification of serum level of lead (3) Significant increase of plasma ammonia and lactate	2013	[14,15]
Adrenoleukodystrophy	X-linked	(1) Medical history collection (2) Physical examination (3) Biochemical findings (4) MRI and MRS of brain (5) USG of abdomen	2010	[16-18]
Bart's syndrome	X-linked	(1) Medical history collection (2) Physical examination (3) Skin biopsy	2012	[19]

(Contd...)

Table 1. (Continued).

Genetic diseases	Inheritance mode	Diagnosis procedure	Publishing year of the first case report	Reference
Bardet-Biedl syndrome	Autosomal recessive	(1) Medical history collection (2) Physical examination (3) Biochemical findings (4) Organ function tests (5) Genital examination (6) Hormone analysis (7) X-ray (8) EKG or ECG	2013	[20-24]
Catecholaminergic polymorphic ventricular tachycardia type 3	Autosomal recessive	(1) Medical history collection (2) Genetic testing	2021	[25]
Cutis laxa syndrome	Heterogeneous	(1) Medical history collection (2) Physical examination (3) Skin biopsy (4) Chest X-ray and echocardiogram	2010	[26,27]
Color blindness	X-linked recessive	(1) Medical history collection (2) Ishihara's test	2009	[28,29]
Cerebral creatine deficiency syndrome	Autosomal recessive or X-linked	(1) Medical history collection (2) MRI of the brain (3) EEG (4) Genetic testing	2021	[30]
Crouzon Syndrome	Autosomal dominant	(1) Medical history collection (2) Physical examination (3) X-ray examination (4) MRI of the brain (5) CT scan of brain and skull	2020	[31]
Congenital hypotrichosis simplex	Heterogeneous	(1) Medical history collection (2) Physical examination (3) Scalp biopsy	2018	[32]
Cockayne syndrome	Autosomal recessive	(1) Medical history collection (2) Physical examination (3) CT scan of the brain (4) EEG (5) Biochemical tests (6) X-ray (7) Genetic testing	2010	[33,34]
Crohn's disease	Single-gene mutation	Not mentioned	2010	[35]
Christmas disease	X-linked recessive	(1) Medical history collection (2) Physical examination (3) Nervous system examination (4) Coagulation factor test (5) Erythrocyte sedimentation rate (6) Hemoglobin test (7) Platelet count blood test	2009	[36]

(Contd....)

Table 1. (Continued).

Genetic diseases	Inheritance mode	Diagnosis procedure	Publishing year of the first case report	Reference
Cystic fibrosis	Single-gene mutation	(1) Answering a questionnaire (2) Medical history collection (3) X-ray (4) Biochemical test (5) Mantoux test (6) ECG (7) Echocardiography (8) Primary Immunodeficiency panel (9) Saccharine test (10) FOB (11) Stool microscopy (12) Sweat chloride test (13) Mutation analysis	2004	[37,38]
Down syndrome	Chromosomal disorder	(1) Physical examination (2) Biochemical test (3) USG (4) Karyotype test	2016	[39,40]
Darier's disease/keratosis follicularis	Autosomal dominant	(1) Medical history collection (2) Biochemical test (3) Histological examination (4) Biopsy	2020	[41]
Duchenne muscular dystrophy	X-linked recessive	(1) Medical history collection (2) Physical examination	2009	[42]
Epidermodysplasia verruciformis	Autosomal recessive	(1) Medical history collection (2) Physical examination (3) Biopsy (4) X-ray	2011	[43]
Edward syndrome	Chromosomal disorder	(1) Medical history collection (2) Transabdominal ultrasound (3) Physical examination (4) Chromosome analysis	2012	[44,45]
Escobar syndrome	Autosomal recessive	(1) Medical history collection (2) Physical examination (3) Evaluation by various specialized doctors (4) Spine radiography	2015	[46]
Ellis-van Creveld syndrome	Autosomal recessive	(1) Medical history collection (2) Biochemical test (3) X-ray	2016	[47]
Fraser syndrome	Autosomal recessive	(1) Medical history collection (2) Physical examination (3) X-ray (4) USG (5) Blood tests (6) Biopsy (7) Karyotype test	2014	[48,49]

(Contd...)

Table 1. (Continued).

Genetic diseases	Inheritance mode	Diagnosis procedure	Publishing year of the first case report	Reference
Familial chylomicronemia syndrome	Autosomal recessive	(1) Medical history collection (2) Physical examination (3) Different blood component tests (4) Coagulation factor test (5) Serum examination (6) Investigation of lipid profile (7) USG (8) Ophthalmoscopy (9) Hemoglobin test (10) Blood sugar analysis (11) Total white blood cell count (12) Investigation of liver and renal function	2015	[50,51]
Familial hypophosphatemic rickets	X-linked dominant	(1) Medical history collection (2) Physical examination (3) X-ray (4) Biochemical examination	2010	[52]
Fanconi anemia	Autosomal recessive	(1) Medical history collection (2) Physical examination (3) Biochemical test (4) USG of abdomen (5) X-ray (6) Karyotype test	2017	[53]
Familial hypercholesterolemia	Autosomal codominant monogenic disease	(1) Medical history collection (2) Physical examination (3) Examination of the cardiovascular system (4) Biochemical test (5) X-ray (6) ECG (7) Echocardiogram	2012	[54,55]
Gaucher disease	Autosomal recessive	(1) Medical history collection (2) Biochemical test (3) X-ray (4) USG (5) Bone marrow examination (6) Leucocyte acid beta-glucocerebrosidase assay (7) Abdominal examination (8) Serum antinuclear antibody and Coombs test (9) Immunochromatographic test strip (10) Endoscopy	2009	[56-58]
Glucose-6-phosphate dehydrogenase deficiency	X-linked recessive	(1) Physical examination (2) Hemoglobin test (3) Hematocrit test (4) Reticulocyte count (5) Chest X-ray (6) Blood smear test (7) Bone marrow examination (8) Cold agglutinins test (9) Coombs test (10) G6PD assay	2017	[59]

(Contd...)

Table 1. (Continued).

Genetic diseases	Inheritance mode	Diagnosis procedure	Publishing year of the first case report	Reference
Huntington's disease	Autosomal dominant	(1) Medical history collection (2) Analysis of blood parameters (3) MRI (4) Neurological examination (5) Genetic testing	2016	[60-62]
Hemophilia	X-linked recessive	(1) Medical history collection (2) Physical examination (3) Coagulation screening test (4) X-ray (5) Biochemical tests	2006	[36,63,64]
Hajdu-Cheney syndrome	Autosomal dominant	(1) Medical history collection (2) Physical examination (3) X-ray	2012	[65]
Hutchinson-Gilford progeria syndrome	Autosomal dominant	(1) Medical history collection (2) Biochemical test (3) Radiographic findings	2017	[66]
Hereditary spastic paraplegia	Autosomal dominant/ recessive/X-linked	(1) Medical history collection (2) Physical examination (3) MRI (4) CT scan	2013	[67]
Inborn errors of metabolism	Autosomal dominant/ recessive/X-linked	(1) Medical history collection (2) Screening of amino acid, organic acid, and fatty acid metabolism disorders by tandem mass spectrometry	2018	[68]
Kartagener's syndrome	Autosomal recessive	(1) Medical history collection (2) Physical examination (3) X-ray (4) Electrocardiogram (5) High-resolution computed tomography	2015	[69,70]
Keratosis follicularis spinulosa decalvans	X-linked recessive	(1) Medical history collection (2) Physical examination (3) Scalp biopsy	2017	[71]
Klinefelter syndrome	Chromosomal disorder	(1) Karyotype test	2018	[72]
Larsen syndrome	Autosomal dominant/ recessive	(1) Medical history collection (2) Physical examination (3) Hematology test (4) Radiological Survey	2015	[73]
Lysosomal storage disorders	Autosomal recessive/X-linked	(1) Clinical evaluation (2) Analysis of urinary metabolites (3) Bone marrow study (4) Liver biopsy	2019	[74]
Marfan syndrome	Autosomal dominant	(1) Medical history collection (2) Physical examination (3) Echocardiography (4) USG (5) Analysis of complete blood count, urine, serum creatinine, and random blood sugar, as well as anti-streptolysin O test (6) Chest radiography	2012	[75-77]

(Contd...)

Table 1. (Continued).

Genetic diseases	Inheritance mode	Diagnosis procedure	Publishing year of the first case report	Reference
Mitochondrial encephalomyopathy, lactic acidosis, and stroke like syndrome (MELAS)	Mitochondrial inheritance	(1) Medical history collection (2) Neurological examination (3) Funduscopy examination (4) Blood lactate test (5) USG (6) EEG (7) CT scan (8) ECG (9) Muscle biopsy	2009	[78]
Myotonic muscle disorders	Autosomal dominant	(1) Medical history collection (2) Physical examination (3) Routine nerve conduction study (4) EMG	2008	[79]
Neurocutaneous syndromes	Autosomal dominant	Examinations of: (1) Major features: Skin, brain, and eye lesions Tumors in the heart, lungs, and kidneys (2) Minor features: Bone cysts, rectal polyps, rectal polyps, dental enamel pits, gingival fibromas, non-renal hamartomas, achromatic retinal patches, confetti skin lesions, and multiple renal cysts	2019	[80]
Noonan's Syndrome	Autosomal dominant	(1) Medical history collection (2) Blood tests (3) Routine urine test (4) IgE test (5) Echocardiogram	2009	[81]
Osteogenesis imperfecta	Autosomal dominant	(1) Medical history collection (2) X-ray	2014	[82]
Osteopetrosis	Autosomal dominant	(1) Medical history collection (2) Physical examination (3) Blood test (4) X-ray (5) USG	1996	[83]
Pachydermoperiostosis	Autosomal dominant	(1) Medical history collection (2) Physical examination (3) Biochemical test (4) X-ray (5) NCV test (6) MRI of the brain (7) Examination of the musculoskeletal system (8) Radiography of the limbs (9) Skin biopsy	2012	[84-86]
Peutz-Jeghers' syndrome	Autosomal dominant	(1) Medical history collection (2) Gastrointestinal examination (3) Blood test (4) OBT (5) USG (6) Upper gastrointestinal endoscopy (7) Barium meal test	2010	[87-89]
Parkinson's disease	Autosomal dominant	(1) Medical history collection (2) Physical examination	2020	[90]

(Contd...)

Table 1. (Continued).

Genetic diseases	Inheritance mode	Diagnosis procedure	Publishing year of the first case report	Reference
Porphyria cutanea tarda	Autosomal dominant	(1) Medical history collection (2) General examination (3) Routine Blood test	2013	[91]
Poland's syndrome	Autosomal dominant	(1) Medical history collection (2) Physical examination (3) Radiological examination (4) X-ray (5) USG (6) Cardiovascular system examination (7) Alimentary system and nervous system examination (8) Echocardiography	2015	[92,93]
Retinitis pigmentosa	X-linked recessive	(1) Medical history collection (2) Neurological examination (3) Fundoscopy	2017	[62]
Spinal muscular atrophy type 3	Autosomal recessive	(1) Medical history collection (2) Nerve examination (3) Biochemical test (4) EMG (5) Muscle biopsy	2019	[94]
Thalassemia	Autosomal recessive	(1) Hemoglobin electrophoresis (2) Complete blood count analysis (3) Bilirubin analysis	2005	[95-97]
Touraine-Solente-Gole syndrome	Autosomal recessive/dominant	(1) Physical examination (2) X-ray	2012	[98]
Turner syndrome	Autosomal dominant	(1) Physical examination (2) Karyotype test	1984	[99,100]
Treacher Collins syndrome	Autosomal dominant	(1) Medical history collection (2) Physical examination (3) Biochemical test (4) X-ray (5) Eye examination	2008	[101]
Thrombophilia	Autosomal dominant/recessive/X-linked	(1) Physical examination (2) CT angiography (3) Biochemical tests (4) Chest radiography (5) Echocardiography (6) Blood coagulation test	2012	[102]
Ulcerative colitis	Multifactorial disorder	(1) Medical history collection (2) Physical examination (3) Stool microscopy (4) Hemoglobin test (5) Serum albumin test (6) Blood test (7) Chest X-ray (8) USG (9) Ileocolonoscopy (10) Colonic biopsy	2013	[103]
Von Hippel-Lindau disease	Autosomal dominant	(1) Medical history collection (2) USG (3) Biochemical test (4) Antibody test (5) Gastrointestinal tract endoscopy (6) CT scan (7) MRI (8) Retinal examination	2012	[104]

(Contd...)

Table 1. (Continued).

Genetic diseases	Inheritance mode	Diagnosis procedure	Publishing year of the first case report	Reference
von Willebrand disease	Autosomal dominant/ recessive	(1) Medical history collection (2) Biochemical tests (3) Endoscopy (4) Bone marrow study (5) von Willebrand factor antigen assay	2019	[105]
Wilson's disease	Autosomal recessive	(1) Medical history collection (2) Physical examination (3) Limb examination (4) Nerve examination (5) Slit-lamp examination (6) ECG (7) CT scan (8) MRI of the brain	2015	[106,107]
Williams syndrome	Autosomal dominant	(1) Medical history collection (2) Physical examination (3) ECG (4) Chest X-ray (5) Cardiac characterization (6) STG aortography (7) Angiography (8) Blood test	2014	[108]
Xeroderma pigmentosum	Autosomal recessive	(1) Medical history collection (2) Physical examination (3) Biochemical test (4) X-ray (5) USG (6) ECG (7) MRI of the brain (8) Skin biopsy (9) Neurological examination	2005	[109-111]

Like many other countries, the prevalence of genetic diseases is progressively increasing in the Bangladeshi population. Out of all the genetic diseases, some are ubiquitous in Bangladesh, such as thalassemia, Down syndrome, and autism spectrum disorder. The most common genetic disease in Bangladesh is beta-thalassemia, and about 7% of the population are carriers^[112]. Over 2000 children are born with thalassemia every year in Bangladesh^[113]. A recent nationwide carrier detection program showed that the overall ratio of beta-thalassemia carrier is 2.24%, whereas the Hb-E trait carrier rate is 8.68%. Besides, the study revealed another concerning factor by screening participants with Hb-D trait, asymptomatic Hb-E disease, suspected Hb-E, beta-thalassemia, hereditary persistence of fetal hemoglobin, and alpha-thalassemia trait, who account for 11.8% carriers with abnormal hemoglobin genes^[114]. Parkinson's disease is another matter of concern as the World Health Organization announced the death rate of Parkinson's disease, which is 0.07% of total deaths in Bangladesh in 2017. The number of deceased individuals is increasing day by day^[115]. Surprisingly, the death rate

jumped to 0.18% of total deaths in 2018^[116]. Different studies have been performed to determine the prevalence rate of autism spectrum disorder in Bangladesh. A national survey in 2013 found that the frequency was 1.55/1000 and 0.68/1000 in different conditions, whereas another cross-sectional study in 2018 found that the prevalence was 0.75/1000 in rural areas^[117]. The Autistic Children's Welfare Foundation presumed that the number of patients with autism spectrum disorder in Bangladesh could be around 300,000, which indicates the significance of taking required steps nationwide to prevent unfortunate consequences^[118]. About 1.3 – 1.5 million cancer patients in Bangladesh and thousands of new patients are diagnosed yearly^[119]. Genetic changes cause most cancers, and nearly 5 – 10% of all cancers are inherited from parents^[119]. Another common genetic disease in this country is cystic fibrosis, caused by mutations in the CF transmembrane conductance regulator protein-coding gene. About one in 2500 babies is born with cystic fibrosis in the United Kingdom, whereas the ratio is 1:31,000 in Asia region^[38]. Although many genetic diseases have already been reported in Bangladesh, their prevalence

data, regional distribution, and mutation profiling are still unknown^[120]. In addition, the number of people diagnosed with genetic diseases, such as attention-deficit hyperactivity disorder, Down syndrome, Turner syndrome, and Klinefelter syndrome, increases^[120]. Besides, nearly eight in 1000 live births in this country suffer from congenital heart defects^[7].

3. The reasons for constructing a human genetic disease database in Bangladesh

Along with the technological advancement in disease identification, distinguishing and reserving the genomic variation such as mutations in different parts of the genome are of great help to control and manage the adverse outcomes of any specific diseases. All genomic data must be deposited in a particular location. That is why different genetic databases have already been constructed, such as Online Mendelian Inheritance of Man (OMIM), Human Gene Mutation Database, and Locus-Specific Databases. Genetic databases gather the molecular genetic data, standardized clinical data, and data regarding associated factors of an individual for the interpretation of gene function, the identification of genes that are mostly present in a specific community, and the differentiation from other communities to estimate the underlying cause of genetic disease^[121]. The broad spectrum of genetic diversity among the people of different countries is the fundamental cause of a diverse array of genetic diseases. Genetic databases help elucidate gene function, estimate the prevalence of genes in populations, distinguish between subtypes of diseases, trace how genes may predispose to or protect against illnesses, and improve medical intervention^[121]. Therefore, a nation-specific genetic disease database should contain information about the diseases' frequency rates and provide clues on the required steps to prevent the spread of the diseases from generation to generation. On realizing the significance, many countries have introduced national databases containing mutation details. However, Asian countries were lagging behind in constructing genetic databases compared to the European countries. To encourage genetic data deposition from Asian countries, the Genome Asia 100K Project has been introduced to assemble population-specific variation data and extend the genome-wide association studies. The GAsP database has already taken DNA sequences from India, Malaysia, Korea, Pakistan, China, and many other Asian countries from their genetic disease databases^[122]. Despite having published case reports on more than 60 genetic diseases, Bangladesh still has not constructed a genetic disease database to collate genetic disease and mutation details. Therefore, the prevalence data and the mutation profiles of most genetic diseases are still unknown, making the diagnosis procedure burdensome^[120,123].

Moreover, the curriculum for a primary medical degree (MBBS) does not cover adequate knowledge in genetics, so medical professionals in rural areas may have limited knowledge and access to the new technologies for detecting a genetic disease^[10]. A genetic disease database should also contain all the possible symptoms of the diseases. The information regarding the possible diagnosis procedures should be added as well. The health-care system faces different problems while managing inherited diseases because of the complexity and heterogeneity of the clinical data. Establishing a genetic disease database will allow extensive access to all the related features of the disease and genetic mutation data that will be beneficial for the precise diagnosis and treatment of patients with genetic diseases. These genetic disease databases serve as a platform to educate health-care professionals, scientists, patients, and the common people of Bangladesh about different genetic diseases and the available treatments.

Moreover, South Asia has the most ethnically diverse population and therefore significant genetic variability compared to the other parts of the world^[113]. Comparing the clinical features of South Asian people with those in the databases of other countries can lead to a false-positive or -negative result because of incompatible mutation patterns. Thus, having a population-specific genetic diseases database with specific mutations can ease the analysis of genetic differences between ethnic groups, trace population diversity, and disease susceptibility. Furthermore, the database will lend a helping hand to the research groups working on human genetic diversity, medical and evolutionary history of ethnic groups, genetic disease diagnosis, and treatment by supplying data while designing study and during result interpretation. Understanding genotype-phenotype correlation, developing molecular diagnostic tests, analyzing mutation, and genetic counseling will be easier with the help of a genetic disease database because all relevant information are in one place. In addition, the database would be helpful for other researchers to analyze specific Asian mutations. Pharmacogeneticists will find the database highly advantageous after utilizing it to understand gene-related variabilities in drug responsiveness and metabolism, thereby facilitating drug screening in accordance with genetic susceptibility before prescribing.

4. Genetic disease database of different Asian countries

The diversity of genetic diseases in different populations depends on geographical location, reproductive practices, and environmental factors. Thus, many neighboring Asian countries have already established their own national genetic disease databases, which are summarized in [Table 2](#).

Table 2. Asian countries and regions having individual genetic disease database.

Country/Region	Name of the database	Link	Reference
Bahrain	National Genome Center (Bio-Bank)	https://www.moh.gov.bh/	[124]
China	China National GeneBank	https://ngdc.cncb.ac.cn/databases	[125]
Dubai	Dubai Genomics	https://www.dha.gov.ae/en	[126]
India	Indian Genetic Disease Database (IGDD)	http://www.igdd.iicb.res.in/	[2]
Indonesia	Genomic Medicine Research Group Database (GMRGDB)	Not available	[127]
Iran	Iranian Human Mutation Gene Bank	www.IHMGB.com	[128]
Israel	Israeli National and Ethnic Mutation Database	http://server.goldenhelix.org/israeli	[129]
Japan	Medical Genomics Japan Variant Database (MGeND)	https://mgend.med.kyoto-u.ac.jp/	[130]
Korea	Korean Mutation Database	http://kmd.cdc.go.kr	[131]
Malaysia	Malaysian Node of the Human Variome Project database (MyHVPDb)	http://hvpmalaysia.kk.usm.my/	[132]
Pakistan	Pakistan Genetic Mutation Database	http://pakmutation.kust.edu.pk/	[133]
Qatar	Qatar Biobank	https://www.qatargenome.org.qa/	[134]
Singapore	The Singapore Human Mutation and Polymorphism Database	http://shmpd.bii.a-star.edu.sg/	[135]
Sri Lanka	Sri Lankan Genome Variation Database	http://www.hgucolombo.org/	[136]
Taiwan	Taiwan Biobank	https://www.twbiobank.org.tw/new_web_en/	[137]
Thailand	Thailand mutation and variation database (ThaiMUT)	http://gi.biotec.or.th/thaimut	[138]
Turkey	Turkish Human Mutation Database	http://hmut-tr.sourceforge.net/	[139]
Vietnam	Vietnamese Genome Research Project	https://www.vinmec.com/en/tin-tuc/hoat-dong-benh-vien/vinmec-announced-its-largest-vietnamese-genome-research-project/	[140]

5. Roadmap to generate a genetic disease database for Bangladesh

Data from authentic sources are a prerequisite for establishing a genetic disease database because the database should consist of details regarding specific genetic mutations, associated protein function alteration, and mutational statistics of different regions. To collect the data, databases such as PubMed, NCBI, OMIM, GeneCard, KEGG, and UniProt will be utilized. Details regarding specific genetic diseases that are scientifically accepted and observed can be obtained from PubMed and NCBI, whereas OMIM, GeneCard, KEGG, and UniProt give a clear dimension of human genes and associated genetic diseases depending on different traits, protein sequences, and functions, along with the correlation between genomic difference and phenotypic expression. Detailed information from all these authentic databases can display an approximate result for every individual when the person has a certain genetic mutation. Obtaining mutational analysis results beforehand will be beneficial in terms of taking

adequate treatment and preventive measures to reduce the chance of disease development. Certain strategies must be considered when making the genetic disease database compatible for captive utilization. The entire procedure of constructing the database can be divided into two broad categories: Data collection and data curation. The overall plan is shown in Figure 1.

5.1. Data collection

The primary data can be obtained from peer-reviewed published articles in various sources, including PubMed and Google Scholar. Signs and symptoms can be added to the database for use by physicians, researchers, and patients. Taking consent from the correspondence before incorporating the data is necessary to determine the accuracy of data and eliminate any further inconvenience. In addition, the user submission option should always be kept open to include all new mutations, signs and symptoms, diagnoses, and treatment procedures and to update the database with helpful information about genetic diseases in Bangladesh. Other sources can be linked, including NCBI, OMIM, KEGG, and PDB, to expand the pieces of information in the database. Geographical

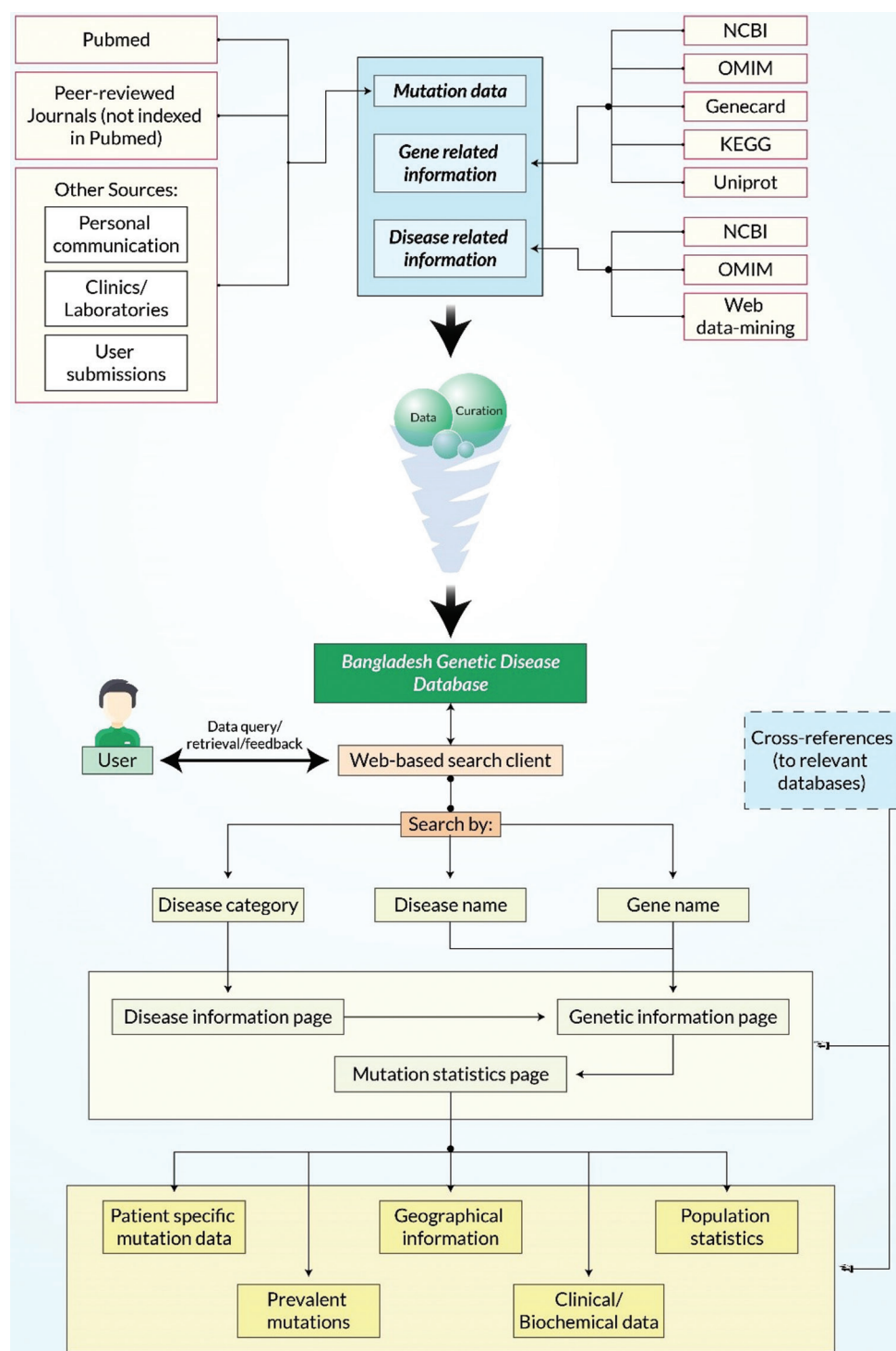


Figure 1. Schematic representation of the construction of proposed Bangladesh Genetic Disease Database.

information, which is vital to researchers to focus on the hotspot genetic disease in a specific community, should be incorporated. Next, the most crucial factor must be included in identifying the causal gene's location in the

chromosome and the accession of nucleotide and protein. Moreover, patient-specific mutation data and symptoms, diagnosis, treatment, and management should also be included.

5.2. Data curation

The database should be updated regularly. Any uploaded mutation information will be cross-checked and filtered before incorporated into the database. Inserting data to a particular place are required, and *in vitro*, *in vivo*, or *in silico* characteristics of the variant will be helpful in this regard, along with differentiating its novelty from others. Mutation data errors will be corrected, modified, or updated whenever needed to maintain accuracy. Data without proper evidence will be removed. In addition, all the data need to be transferred in a single format as per Human Genome Variation Society's recommendation to make the format standardized.

6. Conclusion

The burden of non-communicable diseases increases dramatically throughout the world. Although the genetic data from Bangladesh are scanty, the worldwide prevalence rate of genetic diseases and the 60 published articles found in online sources indicate that Bangladeshi population could be affected by many genetic diseases. A comprehensive genetic disease database will be helpful to provide an insight into the current status of genetic diseases, research, diagnostics, awareness, treatment, and proper patient management. Moreover, a nation-specific genetic disease database would definitely help with strengthening the health-care system.

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Author contributions

Conceptualization: Mohammad Jakir Hosen

Supervision: Mohammad Jakir Hosen and Chunfu Zheng

Visualization: Yusha Araf

Writing - original draft: Yusha Araf and Fariya Akter

Writing - editing and review: Yusha Araf, Saeed Anwar,

Mohammad Jakir Hosen, and Chunfu Zheng

All authors approved the final version of the manuscript.

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CASE REPORT

A novel *USH2A* gene mutation in a family with retinitis pigmentosa: A case report

Xuejiao Li^{1,2}, Haoliang Chen^{1,2}, Na Li^{1,2}, Junhui Wu^{1,2,3} and Yalong Dang^{1,2,3*}

¹Department of Ophthalmology, Sanmenxia Central Hospital, Henan University of Science and Technology, Sanmenxia, Henan, China

²Department of Ophthalmology, Sanmenxia Eye Hospital, Sanmenxia, Henan, China

³Department of Ophthalmology, Henan University of Science and Technology School of Medicine, Luoyang, Henan, China

Abstract

We report a novel variant of the *USH2A* gene in a family affected by retinitis pigmentosa (RP). Genomic DNA was obtained from a 55-year-old woman (the proband) with night blindness who was diagnosed with non-syndromic RP. We identified the compound heterozygous variants c.8559-2A>G and c.151A>T (p.Ile51Phe) in the *USH2A* gene as the underlying cause in the proband; the former variant, instead of the latter variant, has been reported in the literature. The proband's mother carried the c.151A>T variant, while her father and daughter carried the c.8559-2A>G variant. In this family, the proband and her mother developed RP; however, her father and daughter did not develop the disease. Although *in silico* tools predicted that c.151A>T is benign, segregation analysis suggested that this variant could be potentially harmful. The identification of c.151A>T (p.Ile51Phe) variant is a novel finding, and this variant might be a potentially harmful variant of *USH2A* gene. This finding also further expands the mutation spectrum of this gene in the Chinese population.

*Corresponding author:

Yalong Dang
(dangyalong@haust.edu.cn)

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1. Background

Retinitis pigmentosa (RP), a progressive hereditary dystrophic, and degenerative eye disease, occur in approximately 1 in every 3000 – 7000 individuals^[1]. The earliest symptom of this disease is a night blindness, followed by progressive defect of the visual field, eventually decline in the central vision. This disease can be further subdivided into syndromic RP and non-syndromic RP, depending on whether there is defect in the hearing or not.

Usher syndrome, also known as hereditary deafness-RP syndrome, is the most common type of syndromic RP, which is characterized by reduced visual field and visual impairment caused by congenital sensorineural hearing loss and progression of RP^[2]. Usher syndrome can be further subdivided into three types according to the symptom severity: (i) type I that manifests as severe congenital hearing loss, vestibulo-ocular reflex loss, and RP onset during adolescence; (ii) type II that manifests as mild to moderate congenital hearing loss and RP, without vestibular dysfunction; and (iii) type III (rare type) that manifests as progressive hearing loss and RP^[3]. Among them, the type II Usher syndrome is the most common type and is typically caused by mutations in the Usherin

(*USH2A*) gene^[4]. Therefore, this study was conducted to screen and analyze the *USH2A* gene mutations in a Chinese family with RP.

2. Case presentation

2.1. Clinical findings

The family in this study has family members who had developed RP. Including the proband, all family members are of Han Chinese ethnic group, and the age of the proband and her father, mother, and daughter were 55, 89, 84, and 29 years, respectively. The proband, who had an approximately 37-year history of night blindness, was diagnosed with non-syndromic RP, whereas her mother was diagnosed with type II Usher syndrome. In contrast, her father and daughter had no clinical manifestations of RP and presented with a normal phenotype (Figure 1). The proband had a history of night blindness of 18 years with no symptoms of hearing loss. She had a visual acuity level of 0.1 and 0.2 in the right and left eyes, respectively. Fundus photography revealed a yellowish waxy optic disk, thin retinal blood vessels, and proliferation of a large amount of osteocyte-like pigments in the central and peripheral retina. In addition, atrophic changes and proliferative membranes were observed in the macula, as shown in Figure 2A. Optical coherence tomography (OCT) showed the absence of a normal fovea in the macula of both eyes, with the presence of cystoid edema between the layers and hyper-reflective continuum of the epiretinal membrane. Further, both the outer nuclear layer and the nerve fibers around the optic disk were thinned (Figure 2C and E). Pure-tone audiometry subsequently confirmed that her high- and low-frequency hearing abilities were within the normal range, as shown in Figure 2G, with no obvious

abnormalities noted in the vestibular function examination. Therefore, the proband was clinically diagnosed as non-syndromic RP.

Next, the proband's mother had light perception in both her right and left eyes, with yellowish-white opacity of the lens. Furthermore, fundus photography revealed yellowish waxy optic disks, thin retinal blood vessels, proliferation of a large amount of osteocyte-like pigments in the central and peripheral retina, and macular atrophy (Figure 2B). OCT showed the absence of a normal fovea in the macula of both eyes, with the presence of edema between the layers and hyper-reflective continuum of the epiretinal membrane. Further, both the outer nuclear layer and the nerve fibers around the optic disk were thinned (Figure 2D and F). In contrast to the proband, her mother showed sensorineural high- and low-frequency hearing loss in both ears, as shown in Figure 2H according to the pure-tone audiometry examination. However, no obvious abnormality was noted in the vestibular function examination. Thus, the proband's mother was clinically diagnosed as type II Usher syndrome. In contrast, no abnormality was noted in the ophthalmic, vestibular function, and pure-tone audiometry examination in the proband's father and daughter.

2.2. Identification of the *USH2A* gene mutations

Whole-exome sequencing of the genomic DNA of the proband led to the identification of two mutations in the *USH2A* gene, namely, the heterozygous variants c.8559-2A>G and c.151A>T (p.Ile51Phe) (NM_20 6933), which were compared with the UCSC hg19 human genome reference sequence to identify the genetic variations. On average, the mean coverage of the target regions was 182.13X, and for each sample, more than 99.77% of target regions were covered. Sanger sequencing for the identified mutations was performed, and the presence of these two variations in the proband family members was, further, determined and verified. The verification results were consistent with the Illumina sequencing results, as shown in Figure 3. The c.8559-2A>G genetic mutation has been reported previously in multiple studies. Interestingly c.151A>T (p.Ile51Phe) is a novel genetic mutation which was identified for the first time. The c.151A>T (p.Ile51Phe) variant located at chr1-216595528 with a nucleotide switch, that is, A to T at position 151 of the second exon of the *USH2A* gene, results in an amino acid change from isoleucine to phenylalanine at position 51 of the corresponding peptide.

2.3. Pathogenicity analysis of the gene mutations

The c.8559-2A>G variant, known as splice-site mutation, is located at chr1-216051224. It has an allele frequency of

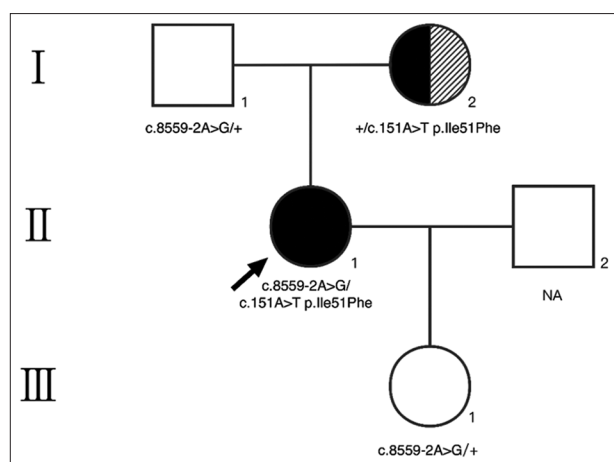


Figure 1. Retinitis pigmentosa pedigree. ■: Male patient; ●: Female patient; □: Healthy male; ○: Healthy female; ♂: Proband; ◐: Retinitis pigmentosa (RP) combined with hearing loss.

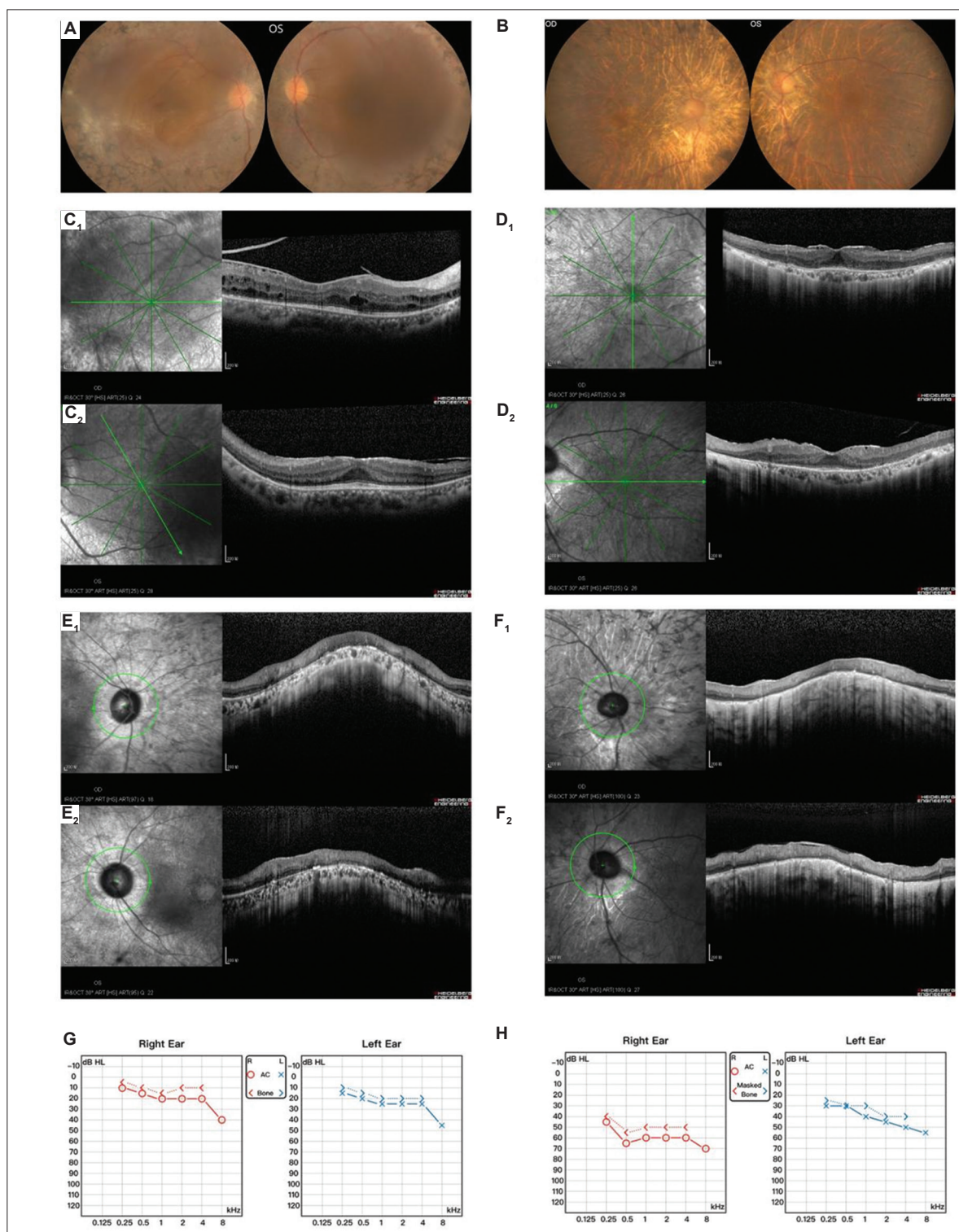


Figure 2. Clinical data of the proband (left) and her mother (right). Fundus photography (A and B), optical coherence tomography (OCT) of the macula (C and D), thickness of the nerve fibers around the optic disk (E and F), and pure-tone audiometry (G and H). The pictures (C₁, D₁, E₁, F₁) represent the right eyes, and pictures (C₂, D₂, E₂, F₂) the left eyes. Fundus photography showed that the optic disk was yellowish and waxy, the retinal blood vessels were thin, and a large number of osteocyte-like pigments proliferated in the central and peripheral retina. OCT of the macula revealed the absence of a normal fovea in both eyes, with cystoid edema between the layers. Hyper-reflective continuum of the epiretinal membrane was visible. Both the outer nuclear layer and the nerve fibers around the optic disk were thinned. Pure-tone audiometry indicated that the hearing of both ears of the proband was within the normal range. In contrast, her mother had hearing loss at both the high- and low-frequencies in both the ears.

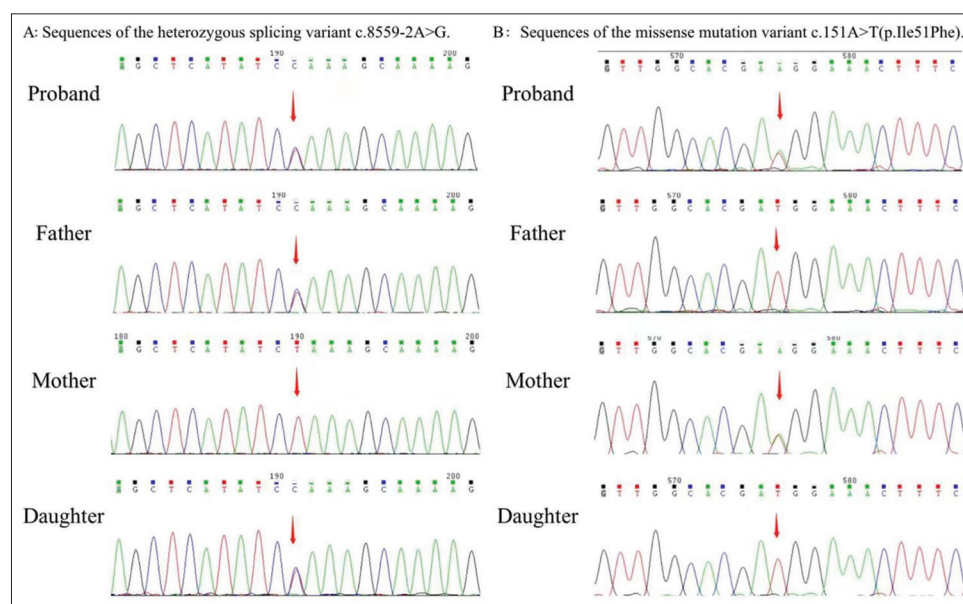


Figure 3. Sanger sequencing confirmation of the variants in *USH2A* identified in this study. Arrows indicate the mutated site.

0.0005 in gnomAD_exome (EAS), 0.0003 in ExAC (EAS), and 0.001 in 1000 genomes. The MutationTaster and Combined Annotation Dependent Depletion (CADD) tools predicted that the mutation was harmful. In addition, the GERP score (++) indicates that the affected amino acids were highly conservative.

In addition, the c.151A>T (p.Ile51Phe) variant was not found in the gnomAD_exome (EAS), ExAC (EAS), and 1000 genomes databases. Sorting Intolerant From Tolerant (SIFT), PolyPhen2, CADD, and Rare Exome Variant Ensemble Learner (REVEL) tools predicted that the novel missense mutation located at chr1-216595528 is benign, while the MutationTaster tool predicted that the variant is a polymorphism, and the GERP score (++) showed that the affected amino acids were non-conservative.

2.4. Segregation analysis

The proband carried both the heterozygous variants, c.8559-2A>G, and c.151A>T (p.Ile51Phe). Although her father and daughter carried c.8559-2A>G variant, neither of them developed RP. In contrast, her mother who carried the novel c.151A>T (p.Ile51Phe) variant suffered from RP. The result of segregation analysis suggested that *USH2A* c.151A>T was consistent with the characteristics of cosegregation (Figure 1).

3. Discussion

In the family of this study, the proband was a 55-year-old woman, who has a history of night blindness with the first symptom started to appear at the age of 18 years. She had no obvious hearing impairment; therefore, she was

clinically diagnosed with non-syndromic RP. Meanwhile, her mother has both RP and hearing loss; therefore, she was clinically diagnosed with type II Usher syndrome. The whole-exome sequencing of the genomic DNA collected from the proband led to the identification of two variants in the *USH2A* gene, with c.151A>T (p.Ile51Phe) being a novel variant of unknown clinical significance, while the c.8559-2A>G variant had been reported in the previous studies^[5,6].

RP can be inherited in autosomal dominant, autosomal recessive, X-linked, or mitochondrial manner^[7]. To date, more than 150 pathogenic gene mutations leading to RP have been discovered. Among these mutations, c.8559-2A>G is the most common variant in the Chinese population^[5,6]. It is a splice-site mutation located in the 42nd intron of the *USH2A* gene^[8]. Researchers found that through its inactivation of splice donor and splice acceptor sites, the c.8559-2A>G variant led to the skipping of exon 43, and may result in the deletion of 41 amino acids, thereby producing pathogenic effect^[9]. In the family of this study, the father and daughter of the proband who carried the heterozygous variant c.8559-2A>G did not show clinical manifestations of RP. The result is consistent with the previous studies, where c.8559-2A>G variant follows an autosomal recessive inheritance pattern, and a heterozygous c.8559-2A>G genotype is not necessarily pathogenic^[8].

In addition, the c.151A>T (p.Ile51Phe) variant located at chr1-216595528 was a novel missense mutation of the *USH2A* gene family. For this variant, the A nucleotide is replaced by T nucleotide at position 151 of the second exon

of the *USH2A* gene, leading to the replacement of isoleucine to phenylalanine at position 51 of the corresponding peptide. SIFT, PolyPhen2, CADD, and REVEL tools predicted that this mutation is benign. In addition, MutationTaster predicted that c.151A>T (p.Ile51Phe) is a polymorphism, whereas GERP score (++) showed that the amino acids were non-conservative. However, it was noted through the segregation analysis that the proband and her mother, who displayed the clinical manifestations of typical RP, carried this novel variant. In contrast, her father and daughter did not carry this mutation, and neither of them developed RP. Therefore, it was concluded that this novel variant is likely to follow an autosomal dominant inheritance pattern. However, there is a limitation in our study, where only Sanger sequencing was used to identify the variants in the proband's mother; therefore, we could not determine whether the mother also carries some other mutations in the *USH2A* gene family.

4. Conclusions

In this report, we describe two heterozygous variants that may potentially affect the development of RP in a Chinese family. The c.151A>T (p.Ile51Phe) variant was identified as a novel mutation of the *USH2A* gene, which could be potentially harmful. The specific mechanism of this variant in the development of RP needs to be further elucidated by future research. The finding of this study further expands the mutation spectrum of the *USH2A* gene in Chinese population.

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Conflicts of interest

All authors have no conflicts of interest to declare.

Authors' contributions

Conceptualization: Yalong Dang

Formal analysis: Haoliang Chen, Na Li, Junhui Wu

Investigation: Xuejiao Li

Writing – original draft: Xuejiao Li, Na Li, Yalong Dang

Writing – review & editing: Yalong Dang, Xuejiao Li

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Sanmenxia Central Hospital. All subjects signed the informed consent forms.

Consent for publication

The subjects had given written consent for the case report to be published.

Availability of data

Data will be available on contacting the corresponding author.

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<https://doi.org/10.1038/jhg.2010.83>

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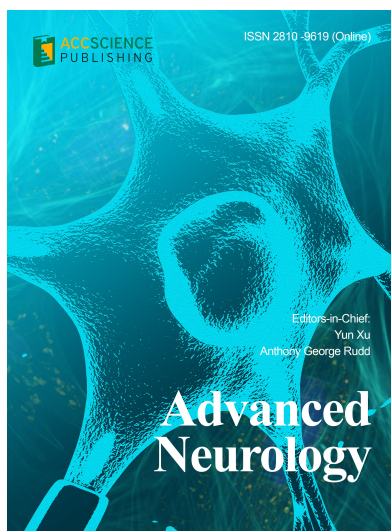
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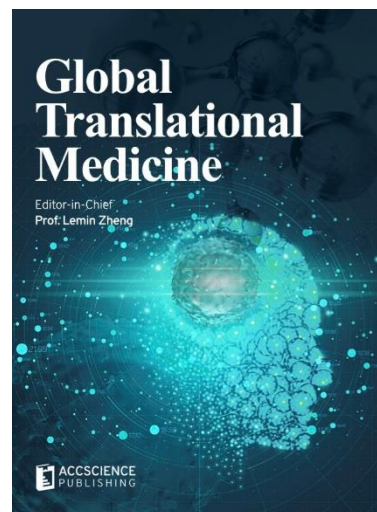
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