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ORIGINAL ARTICLE

Novel insight into *FCSK*-congenital disorder of glycosylation through a CRISPR-generated cell model

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Abstract

Background: *FCSK*-congenital disorder of glycosylation (FCSK-CDG) is a recently discovered rare autosomal recessive genetic disorder with defective fucosylation due to mutations in the fucokinase encoding gene, *FCSK*. Despite the essential role of fucokinase in the fucose salvage pathway and severe multisystem manifestations of FCSK-CDG patients, it is not elucidated which cells or which types of fucosylation are affected by its deficiency.

Methods: In this study, CRISPR/Cas9 was employed to construct an FCSK-CDG cell model and explore the molecular mechanisms of the disease by lectin flow cytometry and real-time PCR analyses.

Results: Comparison of cellular fucosylation by lectin flow cytometry in the created CRISPR/Cas9 *FCSK* knockout and the same unedited cell lines showed no significant change in the amount of cell surface fucosylated glycans, which is consistent with the only documented previous study on different cell types. It suggests a probable effect of this disease on secretory glycoproteins. Investigating O-fucosylation by analysis of the *NOTCH3* gene expression as a potential target revealed a significant decrease in the *FCSK* knockout cells compared with the same unedited ones, proving the effect of fucokinase deficiency on EGF-like repeats O-fucosylation.

Conclusion: This study expands insight into the FCSK-CDG molecular mechanism; to the best of our knowledge, it is the first research conducted to reveal a gene whose expression level alters due to this disease.

KEYWORDS
congenital disorder of glycosylation, CRISPR/Cas9, *FCSK*, fucokinase

Seyed Mohammad Bagher Tabei and Korosh Ashrafi Dehkordi should be considered Joint senior author.

1 | INTRODUCTION

Congenital disorders of glycosylation (CDG), initially identified in 1980 (Jaeken et al., 1980) and still hot in 2023, are a large and rapidly growing group of rare inherited metabolic diseases arising from pathogenic variants in the genes associated with glycosylation (Ng & Freeze, 2018).

Glycosylation is a comprehensive enzymatic process that involves the activation of monosaccharides (glucose, galactose, xylose, N-acetylgalactosamine, N–acetylglucosamin, sialic acid, glucuronic acid, mannose, and fucose) and the subsequent addition of glycans to proteins and lipids through several pathways, including N-linked, O-linked, glycosylphosphatidylinositol (GPI), glycosaminoglycan (GAG) and glycolipids (Varki et al., 2022). Due to the intercellular and intracellular critical roles of this process, it is an important health and disease regulator (Reily et al., 2019).

Fucosylation is a common form of glycosylation that is dependent on guanosine diphosphate L-fucose (GDP-fucose), its specific transporters, and a group of fucosyltransferase enzymes to incorporate L-fucose (6-deoxy-L-galactose) into specific proteins and lipids. Two distinct mechanisms generate GDP-fucose: de novo and salvage pathways. The de novo pathway relies on converting cytoplasmic mannose and glucose to fucose, whereas salvage pathway uses free fucose which is supplied from the environment or recovered from glycoconjugates (Schneider et al., 2017; Figure 1).

It has many biologically relevant functions as involvement in ABO and Lewis^x blood group systems (de Mattos, 2016; Mondal et al., 2018); leukocyte extravasation (Nimrichter et al., 2008); fertilization (Pang et al., 2011); development, particularly neural development (Fenderson et al., 1990; Ohata et al., 2009); immunological modulation (Marth & Grewal, 2008); cancer metastasis (Blanas et al., 2018; Keeley et al., 2019); inflammation (Li et al., 2014; Thompson et al., 1989); and cognitive processes (Mountford et al., 2015; Tosh et al., 2019).

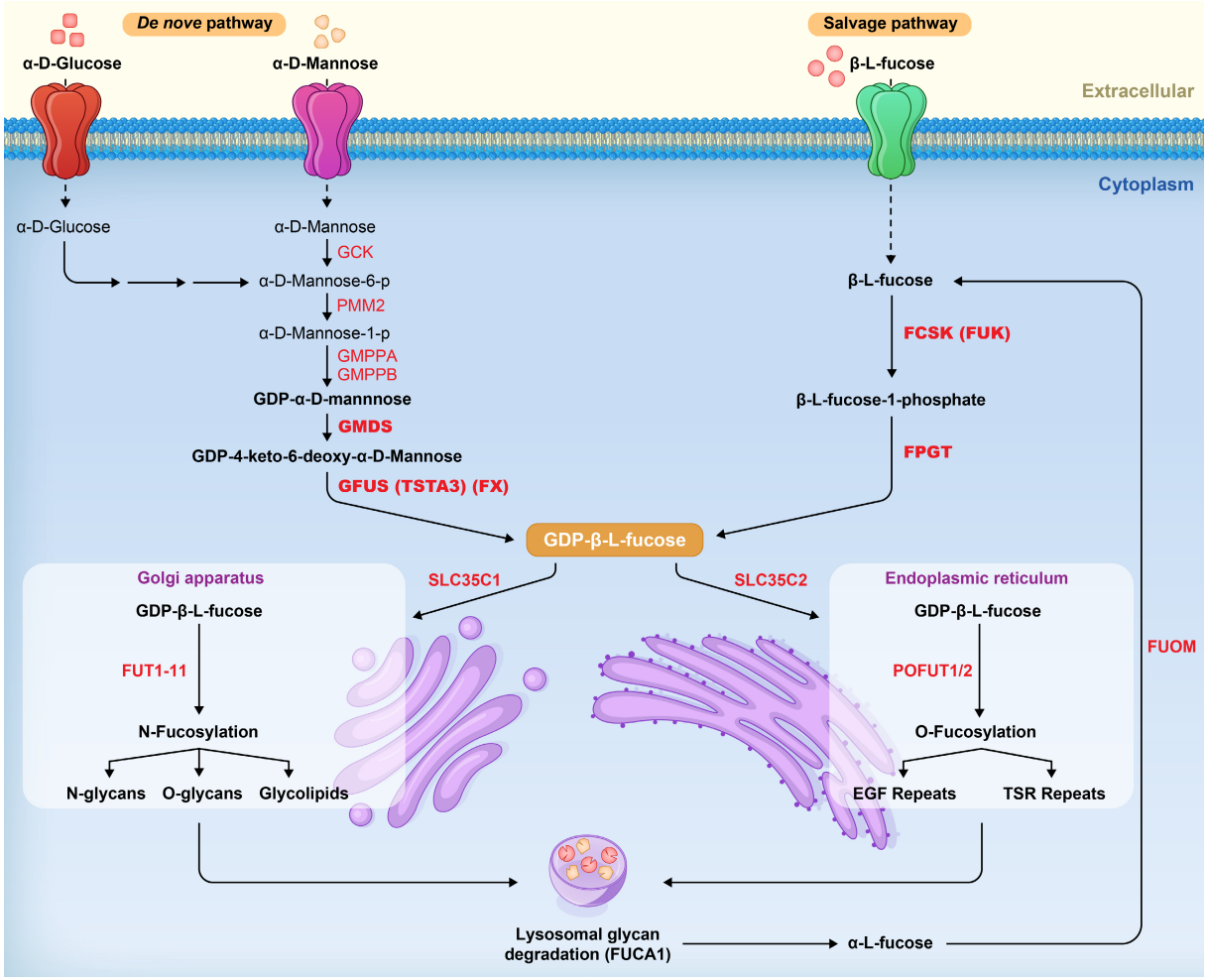

FIGURE 1 The schematic depiction of fucosylation. GDP-L-Fucose synthesis by the de novo and salvage pathways and its utilization in N-fucosylation and O-fucosylation.

Impairment in each fucosylation-related enzyme can lead to CDG with defective fucosylation; although only five types have been described to date, they are named by the official symbol of the impaired gene followed by the suffix "-CDG": SLC35C1-CDG, POFUT1-CDG, FUT8-CDG, FCSK-CDG, and GFUS-CDG (Hullen et al., 2021).

FCSK-CDG, congenital disorder of glycosylation with defective fucosylation 2 (CDGF2), MIM# 618324, is an autosomal recessive genetic disease resulting from pathogenic variants in *FCSK* (MIM# 608675), the gene that encodes fucokinase (EC 2.7.1.52), an essential enzyme in the salvage pathway (Ishihara et al., 1968; Stelzer et al., 2016; see Figure 1). This extremely rare disease was first characterized in 2018 by Ng et al. in two unrelated patients with severe multisystem manifestations such as brain anomalies, intellectual disability, developmental delay, intractable seizures accompanied by epileptic encephalopathy, muscle contractures, hypotonia, walking disability, poor feeding, difficulty in gastric emptying, and ocular disorders (Ng, Rosenfeld, et al., 2018). Subsequently, three other patients with different pathogenic variants in the *FCSK* gene have been reported, including one by our team (Al Tuwaijri et al., 2023; Manoochehri et al., 2022; Ozgun & Sahin, 2022). Table 1 displays the characteristics of all FCSK-CDG patients documented thus far (Al Tuwaijri et al., 2023; Manoochehri et al., 2022; Ng, Rosenfeld, et al., 2018; Ozgun & Sahin, 2022).

FCSK-CDG patient samples and the disease cell model in Ng et al.'s study, despite the significant decrease in fucokinase, have shown no detectable fucosylation change (Ng, Rosenfeld, et al., 2018). It was thought that the de novo pathway generated the majority of GDP-fucose (Ng, Rosenfeld, et al., 2018; Yurchenco & Atkinson, 1975, 1977); however, given the vast abnormalities in FCSK-CDG patients, accepting the minimal or negligible impact on cellular fucosylation due to loss of salvage pathway is challenging. However, recent insight into fucosylation support the importance of the salvage pathway (Lau et al., 2015; Smith et al., 2002; Sosicka et al., 2020, 2022).

In this study, we employed CRISPR/Cas9 system to construct a human U-87 MG cell line model of FCSK-CDG disease to survey the cellular fucosylation in the absence of the salvage pathway. The utilized cell line, glioblastoma, was selected considering the putative cell type-dependent contribution of salvage pathway in fucosylation (Ng, Rosenfeld, et al., 2018; Sosicka et al., 2022) and due to prevalent brain anomalies and neurological manifestations of FCSK-CDG patients.

2 | MATERIALS AND METHODS

2.1 | Ethical compliance

This study was approved by the ethics committee of Shahrekord University of Medical Sciences (IR.SKUMS.REC.1400.250).

2.2 | Guide RNAs design and cloning

Utilizing four online design tools, CHOP CHOP (http://chopchop.cbu.uib.no), CRISPOR (http://crispor.tefor.net), Cas-Designer (http://www.rgenome.net/cas-designer/), and CRISPick (http://portals.broadinstitute.org/gppx/crispick//public), and based on the *FCSK* gene sequence (Ensembl ID ENSG00000157353.17, 16: 70454595–70480274), we designed two guide RNAs (gRNAs) to target both sides of the gene and delete almost its entire length. The following gRNAs were used in this study:

Guide 1 with PAM: 5'-TCACACGAACCTATCTC CGGAGG −3′ (Intron 1; forward strand).

Guide 2 with PAM: 5'-ATCATACGCCGCACAGT CAGGGG-3′ (Exon 23; reverse strand).

Each gRNA was inserted into the pSpCas9 (BB)-2A-GFP (PX458) vector using BbsI restriction enzyme (Thermo Fisher Scientific). The plasmid constructs were transformed into separate *DH5α* competent *Escherichia coli* (*E. coli*) cells and extracted using Plasmid Extraction Maxi Plus Kit (Favorgen) after increase through bacterial culture.

2.3 | Validation of the cloned guides

To verify the correct insertion of each gRNA into the PX458 vector, we performed PCR, and the PCR product was evaluated by Sanger sequencing.

The used primers were as follows: Forward, 5'-TTCTTGGGTAGTTTGCAGTTTTAA-3′ and Reverse, 5′-CACGCGCTAAAAACGGACTA-3′ (Dara et al., 2021).

2.4 | Cell culture

U-87 MG (Uppsala 87 malignant glioma) human cell line was purchased from Pasteur Institute of Iran and cultured in a DMEM-F12 medium including 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Thermo Fisher Scientific), at 37°C with 5% CO_2.

TABLE 1 Molecular and clinical characterization of CDGF2 patients (Al Tuwaijri et al., 2023; Manoochehri et al., 2022; Ng, Rosenfeld, et al., 2018; Ozgun & Sahin, 2022).

ID		Patient 1	Patient 2	Patient 3[a]	Patient 4	Patient 5[a]
Age		6 years	7 years	18 months	4.5 years	2 years
Age at onset of symptoms		3 years	4 years	Infancy	Infancy	Infancy
Sex		Male	Female	Female	Male	Male
Descent		Hispanic	Qatari (Middle Eastern)	Somalian	Iranian (Middle Eastern)	Saudi (Middle Eastern)
FCSK pathogenic variant(s)		c.667T>C, p.Ser223Pro; c.2047 C>T, p.Arg683Cys	c.2980 A>C, p.Lys994Gln	c.993_1011del, p.Glu335Hisfs*55	c.379 C>A, p.Leu127Met; c.394G>C, p.Asp132His	c.3013G>C, p.Val1005Leu
Mutation type		Compound heterozygous	Homozygous	Homozygous	Homozygous	Homozygous
Consanguinity		No	Yes	No	Yes	Yes
Pregnancy complications		No	Born premature at 2 weeks (10 g)	No	No	No
Intellectual disability	4/5	Severe	Severe	Mild	Severe	No
Developmental delay	4/5	Yes	Yes	Yes	Yes	No
Gait problems	4/5	Nonambulatory	Nonambulatory	Nonambulatory	Nonambulatory	No
Hypotonia	4/5	Central hypotonia	Central hypotonia with spasticity of extremities	Central hypotonia	Central hypotonia	No
Abnormalities in brain MRI	4/5	Corpus callosum dysplasia and delay in myelination of deep white matter	Atrophy of cerebellum, corpus callosum agenesis, abnormalities in white matter presented with severe periventricular leukomalacia and paucity of white matter	Periventricular white matter hyperintensities, enlargement of ventricle with encephalomalastic areas	Hypoplasia of cerebellar and vermis with racing car sign, corpus callosum dysplasia	Not available
Seizures/Epilepsy	4/5	Generalized tonic–clonic seizures due to multifocal epileptic activity in the bilateral hemispheres	Seizures with hypsarrhythmia in EEG and initially consistent with infantile spasms	No	Unprovoked generalized tonic–clonic seizures	Infantile spasms
Speech disorder	1/5	No	No	No	Yes	No
Ocular disorders	4/5	Symmetric maculopathy with severe visual dysfunction	Optic nerve atrophy strabismus, cortical blindness, nystagmus	Cortical blindness	Complete loss of vision	No
Dysmorphic face	0/5	No	No	No	No	No
Feeding problems	3/5	Yes	Yes	No	Yes	No
Hepatopathy	1/5	Elevated gamma-glutamyl transferase (GGT)	No	No	No	No

TABLE 1 (Continued)

ID		Patient 1	Patient 2	Patient 3[a]	Patient 4	Patient 5[a]
Contractures	3/5	Yes	Yes	No	Yes	No
Stature	0/5	Normal for age	Normal for age	Normal for age	Normal for age	Normal for age
Respiratory problems	3/5	Recurrent respiratory infections	Respiratory difficulties	No	Respiratory difficulties	No
Recurrent infections	2/5	Yes	No	No	Yes	No
Reference		Ng, Rosenfeld, et al. (2018)	Ng, Rosenfeld, et al. (2018)	Ozgun and Sahin (2022)	Manoochehri et al. (2022)	Al Tuwaijri et al. (2023)

[a]It is notable that these patients' mild clinical condition is probably due to their young age, as the onset of symptoms was at 3 and 4years in patients 1 and 2, respectively.

2.5 | Transfection and fluorescence-activated cell sorting (FACS)

U-87 MG cells were seeded in a 6-well cell culture plate, and 80% confluency was achieved after 24 h; they were co-transfected by guide-1 and guide-2 CRISPR/Cas9 constructs utilizing Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instruction. In the subsequent 2–8 h, the cells were examined by fluorescence microscopy.

After 48 h, FACS was employed by FACSAria™III (BD Bioscience) to select the transfected cells due to GFP expression in PX458 constructs; GFP-positive cells were collected and cultured for post-transfection analyses.

2.6 | Single-cell isolation, DNA extraction, and PCR-based analyses

To acquire *FCSK* homozygous knockout (KO) cells (monoclonal cell population), serial dilution was done on a population of transfected cells to reach 10 cells/mL concentration; a 100 μL aliquot of this cell suspension was added to each well of a 96-well plate, one cell per well; single cells were incubated and the plate was scanned regularly to transfer the single cells that had been proliferated to a larger culture plate, one by one. 96 well plate → 24 well plate → 6 well plate.

Genomic DNA of each monoclonal cell population was extracted (Blood/Cultured Cell Genomic DNA Extraction Mini Kit, Favorgen) and evaluated by performing multiplex PCR with two sets of primers, set #1 and set #2, amplifying wild-type and CRISPR-edited DNA, respectively.

Primer set #1: Forward primer (5′-GGCTCC TTGGTCAACAGATAGA-3′, located in exon 23 downstream of Guide 2), Reverse primer (5′-AAGGTGGAGGTAGAAGAGGTCA-3′, located in exon 21).

Primer set #2: Forward primer (5′-GGCTCCTTG GTCAACAGATAGA-3′, located in exon 23 downstream of Guide 2), Reverse primer (5′-AAATCACA CCCATCCACATTTT-3′, located in intron 1 upstream of Guide 1).

To confirm the desired deletion, Sanger Sequencing was conducted on the obtained *FCSK* homozygous deleted cells' genomic DNA PCR product.

2.7 | Quantitative real-time RT-PCR assay

Total RNA was extracted from *FCSK* KO and unedited U-87 Cells using Blood/Cultured cell total RNA

Purification Mini Kit (Favorgen) and reverse transcribed into cDNA by PrimeScript™ 1st strand cDNA Synthesis Kit (Takara Bio). cDNA amplification to detect expression levels of *FCSK* (NM_145059.3), *NOTCH3* (NM_000435.3), and *ACTB* (NM_001101.5) was performed by RT-PCR using SYBR green and specific primers on a Quantstudio™ 3 system (Thermo Fisher Scientific). The results were normalized to the reference housekeeping gene *ACTB* and calculated using the $2^{-\Delta\Delta CT}$ method. Each experiment was performed in triplicate. The used primers are listed in Table 2.

2.8 | Western blot assay

To study *FCSK* expression at the protein level, we extracted the total protein of the *FCSK* KO and unedited U-87 cells, separated by SDS PAGE; subsequently, they were transferred to the PVDF (polyvinylidene difluoride) blotting membrane and blocked with 2% non-fat milk in TBST (tris-buffered saline with Tween-20) buffer for 75 min at room temperature. Then, the membranes were incubated with fucokinase antibody (Fucokinase (F-9): sc-377371, 1:300) (Santa Cruz Biotechnology), as the primary antibody, at 4°C for 1 h. The membranes were washed and incubated with mouse anti-rabbit IgG-HRP: sc-2357, 1:1000 (Santa Cruz Biotechnology), horseradish peroxidase-conjugated secondary antibody, for 75 min at room temperature. Following another washing step, protein bands were visualized using the enhanced chemiluminescence reagent. ß-actin antibody (ß-Actin (C4): sc-47778, 1:300) (Santa Cruz Biotechnology) was used as the internal control.

2.9 | Lectin flow cytometry assay

Unedited U-87 and *FCSK* KO U-87 cells were centrifuged; each cell pellet was resuspended in PBS, aliquoted, and incubated with 10 µg/mL AAL-FITC (Vector Laboratories), or 70 µg/mL UEA I-FITC (Vector Laboratories) for 30 min in 4°C and protected from light. They were further washed twice, resuspended in PBS, and subjected to flow cytometry. As running a negative control in parallel with each assay, lectins were preabsorbed with 300 Mm L-fucose (Sigma-Aldrich), substituted into the procedure in place of the unabsorbed lectin, and incubated under the same conditions. All analyses were done using BD FACSCalibur (BD Biosciences) flow cytometer and BD CellQuest Pro software (BD Biosciences).

2.10 | Statistical analysis

The results of each assay, which was performed in triplicate, were analyzed in GraphPad Prism software (version 9; GraphPad Prism) using the student's *t*-test and displayed as mean ± standard deviation (SD). *p*-value <0.05 was considered statistically significant.

3 | RESULTS

3.1 | Cloning

After cloning and bacterial culture, *E.coli* DH5α colonies carrying plasmid constructs appeared in Amp^+ LB agar plates due to PX458's Amp resistance marker. Engineered colonies containing each gRNA were detected by observation of the 241 bp PCR product in colony PCR, and Sanger sequencing confirmed the correct insertion of gRNAs into PX458 vectors (Figure 2).

3.2 | Transfection, single-cell isolation, and PCR-based analyses

48 h after transfection, the transfected cells were observed by fluorescence microscope; then, we sorted and collected the cells by FACS. Multiplex PCR on the total genomic DNA extracted from the transfected cells population detected CRISPR-edited DNA by amplifying the desired 217 bp product in addition to the 1012 bp one, which was the only detected amplified product in the untransfected

TABLE 2 Primers for real-time PCR.

Targeted gene	Primers sequence		Product size (bp)
ACTB (Ramezani, 2021)	Forward	5′-GCCTTTGCCGATCCGC-3′	90 bp
	Reverse	5′-GCCGTAGCCGTTGTCG-3′	
FCSK	Forward	5′-CCTCGTTGGCCGTCTGGA-3′	93 bp
	Reverse	5′-CGAACACCTGTCCTCTTGAAGAAT-3′	
NOTCH3	Forward	5′-GGCATCAACCGCTACGACTG-3′	117 bp
	Reverse	5′-CCCATCCACACAGGAACCTC-3′	

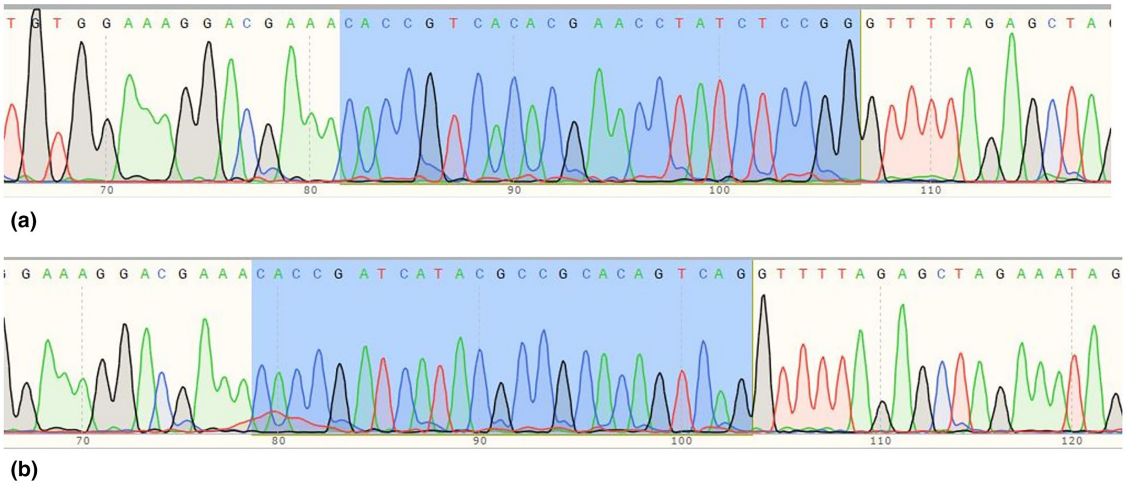

FIGURE 2 Sanger sequencing results indicating correct guide RNAs cloning into PX458 vectors using BbsI restriction enzyme. Highlighted 25 nucleotides include overhang complementary to the overhang in digested PX458 (5′-CACC), an extra G nucleotide to promote optimal expression by the hU6 promoter expressing the gRNA in PX458, and the 20 nucleotide gRNA. (a) Electropherogram of inserted Guide 1 in PX458 vector. (b) Electropherogram of inserted Guide 2 in PX458 vector.

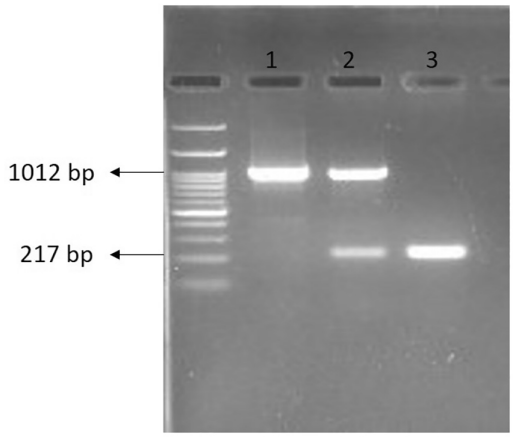

FIGURE 3 PCR Products of 1012 bp and 217 bp on agarose gel electrophoresis indicating wild-type and CRISPR-edited DNA, respectively. 1: Untransfected U-87 cells, 2: Transfected U-87 cells population, 3: Homozygous CRISPR-edited U-87 cells.

negative control U-87 cells. In the case of the *FCSK* homozygous knockout cells, obtained by means of single-cell isolation, only the 217 bp product was amplified, because of having no wild-type DNA (Figure 3). Sanger sequencing confirmed the fine cutting of the designed positions on *FCSK* gene intron 1 and exon 23 by cas9, 17,341 bp deletion, and joining cut sites (Figure 4).

3.3 | Real-time RT-PCR and western blot analyses

RT-PCR and western blot assays confirmed *FCSK* KO as no *FCSK* expression was observed at the mRNA or protein levels in CRISPR-edited U-87 cells, whereas it was detected at both levels in unedited U-87 cells. The result of Western blot is shown in Figure 5a.

Real-time RT-PCR analysis of *NOTCH3* mRNA expression revealed a significant decrease in the *FCSK* KO U-87 cells compared with unedited cells (0.26 ± 0.02 fold change; p-value <0.0001) after normalization to a control (Figure 5b).

3.4 | Lectin flow cytometry analyses

The mean of MFIs (mean fluorescence intensity) in the *FCSK* KO versus unedited U-87 cells analyzed by lectin flow cytometry were 407.55 ± 55.72 vs.463.58 ± 85.47 and 23.87 ± 3.53 vs. 26.91 ± 1.49 in AAL and UEA I staining, respectively; there was no statistically significant change in the cell surface fucosylation. Figure 6 represents overlay histograms of lectin-binding in unedited and *FCSK* KO U-87 cells.

4 | DISCUSSION

In 2018, Ng et al. described *FCSK*-congenital disorder of glycosylation as the first genetic disease identified specifically within the de novo or salvage pathways of fucosylation (Ng, Rosenfeld, et al., 2018); however, FUT8-CDG, SLC35C1-CDG, and POFUT1-CDG, as disorders due to fucosylation related to downstream components of its pathway, had already been identified (Basmanav et al., 2015; Li et al., 2013; Lübke et al., 2001; Luhn et al., 2001; Ng, Xu, et al., 2018).

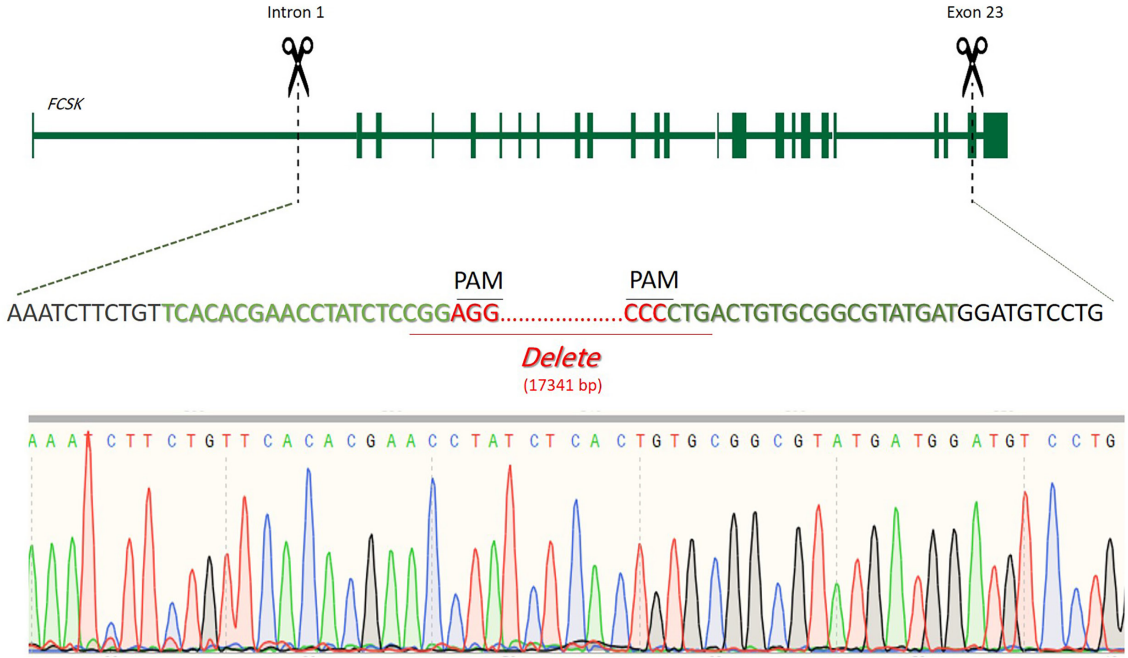

FIGURE 4 Schematic depiction of the CRISPR/Cas9 targeted sequence and the electropherogram of that region in the CRISPR-edited U-87 cells. The double cuts in Intron 1 and Exon 23 resulted in 17,341 bp elimination from the *FCSK* gene.

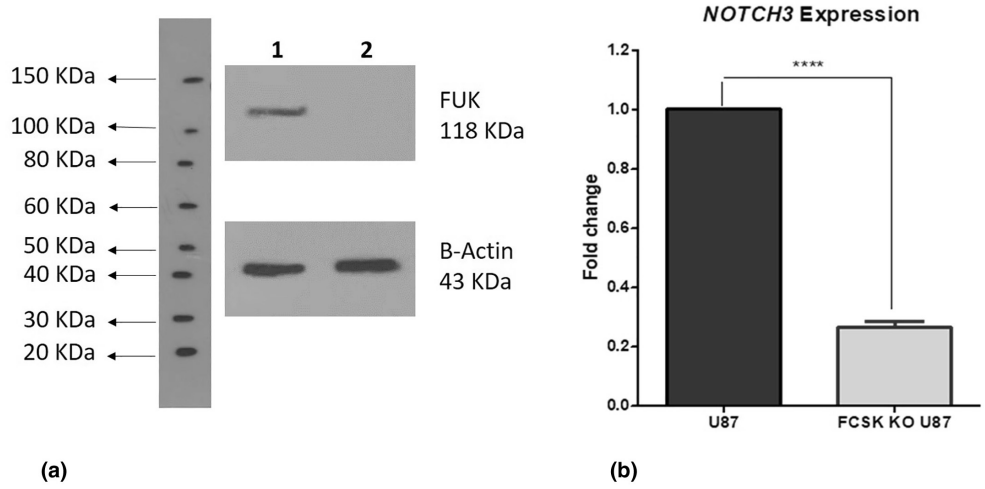

FIGURE 5 The results of Western blot and Real-time PCR assays. (a) Western blot analysis confirms *FCSK* Knockout; lanes 1 and 2 are untransfected U-87 cells, and homozygous CRISPR-edited U-87 cells (*FCSK* Knockout), respectively. (b) Demonstrates a significant decrease in the *NOTCH3* expression in the *FCSK* KO compared to unedited U-87 cells based on real-time PCR analysis. ****: p-value < 0.0001, Bars and error bars represent mean ± SD.

Despite the identification of FCSK-CDG (Ng, Rosenfeld, et al., 2018) and GFUS-CDG (Feichtinger et al., 2021), as well as investigation of the involvement of de novo pathway enzymes GMDS and TSTA3 (GFUS) and salvage pathway enzyme FUK (FCSK) in modifying the invasive and metastatic features of several types of cancers in the last decade (Keeley et al., 2018; Kizuka et al., 2017; Lau et al., 2015; Nakayama et al., 2013; Wei et al., 2018), the specific contribution of each of the two distinct fucosylation pathways has not been well elucidated yet.

According to studies conducted in the mid-1970s, it was thought that the de novo pathway supplied over 90% of GDP-fucose and that less than 10% came from salvage (Yurchenco & Atkinson, 1975, 1977). Given that despite their meticulous work, studies were done only in a single cell line and with a single fucose concentration, and rethinking old concepts based on recent studies, a more

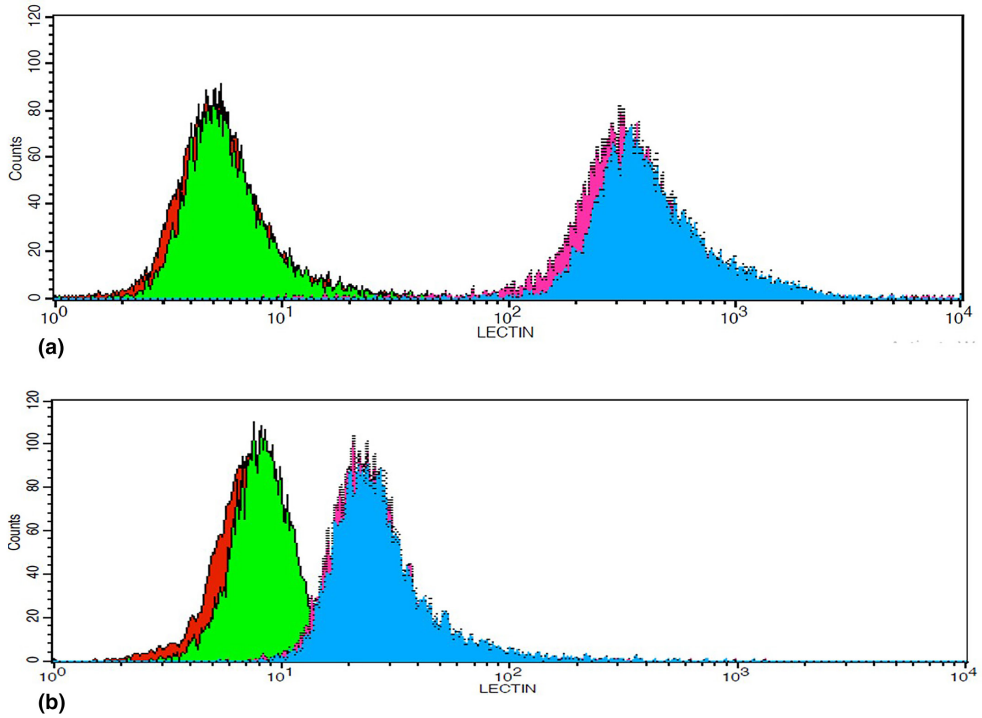

FIGURE 6 The results of lectin flow cytometry analyses indicate no significant difference between unedited and *FCSK* KO U-87 cells in AAL-binding nor UEA I-binding. (a) A representative overlay histogram of AAL-binding in unedited and *FCSK* KO U-87 cells. (b) A representative overlay histogram of UEA I-binding in unedited and *FCSK* KO U-87 cells. In both (a and b) Green: Unedited U-87 cells stained by preabsorbed AAL-FITC (a) or UEA I-FITC (b) as a negative control. Red: *FCSK* KO U-87 cells stained by preabsorbed AAL-FITC (a) or UEA I-FITC (b) as a negative control. Blue: Unedited U-87 cells stained by AAL-FITC (a) or UEA I-FITC (b). Pink: *FCSK* KO U-87 cells stained by AAL-FITC (a) or UEA I-FITC (B).

considerable contribution to fucosylation is suggested for the salvage pathway (Lau et al., 2015; Sosicka et al., 2020). A recent study indicated a comparable contribution of salvage and de novo pathways in the fucosylation of HepG2 and Huh7 cells (Sosicka et al., 2022).

Although Ng et al. have confirmed the significant decrease of fucokinase, *FCSK*-encoded protein, in patients and the created CRISPR/Cas9 *FCSK* knockout HAP1 cell line, they have not seen any detectable fucosylation change in the fibroblast and serum samples of patients, and *FCSK* knockout cells (Ng, Rosenfeld, et al., 2018). Their two reported cases and the subsequent reported ones, FCSK-CDG patients, manifested intellectual disability, developmental delay, seizure, epilepsy, gait problems, hypotonia, contractures, abnormal brain MRI, feeding difficulty, and ophthalmological disorders (Al Tuwaijri et al., 2023; Manoochehri et al., 2022; Ng, Rosenfeld, et al., 2018; Ozgun & Sahin, 2022).

Probable cell type-dependent contribution of salvage and de novo pathways in fucosylation is a notion that can reveal the reason for this observed unchanged fucosylation despite such severe manifestations due to fucokinase defect; in other words, although some cell types may require the salvage pathway critically, others can function well without it (Ng, Rosenfeld, et al., 2018; Sosicka et al., 2022). We hypothesized that brain cells were presumably dependent on the salvage pathway due to important prevalent neurological manifestations of FCSK-CDG patients, and selected the U-87 MG cell line to create a new cell model of this disease.

Another notable notion is the distinct preference between salvage and de novo pathways-originated GDP-fucose among various fucosyltransferases (Keeley et al., 2018; Lau et al., 2015; Sosicka et al., 2022). N-glycosylation performs in Golgi apparatus by the use of 11 known fucosyltransferases (FUTs): FUT1,2 are responsible for adding fucose to the terminal galactose of glycans in α1-2 orientation; FUT3-7 and FUT9-11 mediate α1-3 and α1-4 fucosylation of terminal GlcNAc in glycans; and FUT8, the only core fucosylation enzyme, adds fucose to the initial GlcNAc residue on N-glycans in α1-6 linkages (Becker & Lowe, 2003). Suggesting an inhibitory role on melanoma invasion and metastasis for fucose salvage pathway by inhibition of invadopodia formation, Keeley et al. indicated that this inhibitory effect was mediated through α1-2 fucosylation, but not the α1-3 or α1-4; they propose that although GDP-fucose is the fucosylation donor for all fucosyltransferases,

salvage pathway-originated GDP-fucose might be preferentially provided for certain fucosyltransferases (Keeley et al., 2018). According to a recent study, each fucose linkage added by distinct fucosyltransferase, situated in separate Golgi compartments, depends on different GDP-fucose pools since they have different access to salvage or de novo pathways-originated GDP-fucose (Sosicka et al., 2022).

Given the latter notion, investigating fucose in different linkages was our second assumed key to the mystery. Lectins are carbohydrate-binding proteins whose affinity to particular carbohydrates can be as specific as that of antibody–antigen or enzyme-substrate. Regarding this potency, they are being widely used to recognize specific carbohydrate complexes in cellular glycoproteins and glycolipids. (Minko, 2004) We utilized two lectins, not only AAL as the most common fucose-specific lectin that preferentially recognizes α1–6 and α1–3 linked fucose while recognizing all types of N-linked fucose with variable efficiencies but also UEA I which prefers (α1-2) linked fucose residues (Sosicka et al., 2022).

Comparing cellular fucosylation by AAL and UEA I lectin flow cytometry in the created CRISPR/Cas9 *FCSK* knockout and the unedited U-87 MG cell lines showed no significant change in cell surface glycoproteins and glycolipids. It can be probably assumed that the salvage pathway preferentially provides GDP-fucose for secretory glycoproteins since our data showed unchanged cell surface fucosylation in another *FCSK* KO cell line; on the other hand, HepG2 and Huh7, two cell lines that have previously shown comparable contribution of salvage and de novo pathways by GC–MS, are both professional secretory cells. (Sosicka et al., 2022) However, it is notable that lectin flow cytometry showed low amounts of α1-2 fucosylation on the cell surface of our created model; according to the results and suggestions of previous studies (Keeley et al., 2018; Lau et al., 2015; Sosicka et al., 2022); well-documented involvement of α1-2 fucose in neuronal growth and development molecular mechanisms (Kalovidouris et al., 2005; Mountford et al., 2015; Tosh et al., 2019); and prevalent brain anomalies and neurological manifestations of FCSK-CDG patients, it seems that this type of fucosidic-linkage is predominantly incorporated in secretory glycoproteins. After all, fucosylation, as a glycosylation type, is too complex to be elucidated clearly; some have termed glycobiology "glycophobia" due to longevity and hesitation of working on it (Ng & Freeze, 2018). However, the contribution of cytoplasmic GDP-fucose to fucosylation process is determined by its heritage, and the cells possess the ability to distinguish GDP-fucose of different origins (Sosicka et al., 2022).

Next, we investigated the *NOTCH3* gene expression, as a potential target of O-fucosylation (Schneider et al., 2017). O-fucosylation, occurring in the endoplasmic reticulum, is carried out by two known GDP-fucose protein O-fucosyltransferase (POFUT) enzymes: POFUT1 attaches fucose to Ser/Thr residues in epidermal growth factor (EGF)-like repeats, while POFUT2 attaches fucose to Ser/Thr residues in ThromboSpondin type 1 Repeats (TSRs) (Schneider et al., 2017). Notch proteins are among more than 100 proteins that have EGF-like repeats required for POFUT1 fucosylation. In mammals, the Notch signaling pathway has four Notch receptor proteins (Notch1-Notch4) and five Notch ligands (JAG1, JAG2, DLL1, DLL3, and DLL4) (Stahl et al., 2008). This important conserved signaling pathway influences an amazing range of cell fate decisions and is involved in so many developmental and biological processes that it is impossible to think of a tissue or growing organ that does not rely on it at some point (Sachan et al., 2023). Notch fucosylation, as an important post-translational modification, is implicated in its receptor-ligand binding (Rana & Haltiwanger, 2011). Subsequent to ligand-mediated activation of Notch receptors, they undergo a series of proteolytic cleavages, and the Notch intracellular domain (NICD) translocates to the nucleus. Within the nucleus, NICD constitutes a complex with DNA-binding protein RBPJ and transcriptional co-activator mastermind; this transcription activation complex regulates the expression of Notch pathway target genes, including Notch receptors and ligands genes themselves. In the absence of NICD in the nucleus, RBPJ interacts with multiple transcriptional repressors and represses the transcription of target genes (Bocci et al., 2020; Bray, 2016).

Sosicka et al. in a recent study on the Huh7 cell line have indicated that POFUT1 relies more on salvage pathway-originated GDP-fucose, compared with POFUT2, which prefers the GDP-fucose of the de novo pathway (Sosicka et al., 2022). The RT-PCR assay revealed a significant decrease in *NOTCH3* expression level in our *FCSK* KO U-87 cells compared with the same unedited cells. This reduced expression level of the *NOTCH* gene indicates reduced Notch signaling activity due to defects in Notch protein fucosylation; this result not only proves the contribution and importance of the salvage pathway in O-fucosylation but also is compatible with Sosicka et al.'s findings (Sosicka et al., 2022).

As to clinical phenotypes, the majority of over 30 reported patients with autosomal recessive inherited CDGs with defective fucosylation (SLC35C1-CDG, FUT8-CDG, FCSK-CDG, and GFUS-CDG) had similar manifestations as developmental delay, intellectual disability, feeding problems, hypotonia, and abnormal brain MRI (for those whose data were available). Short stature and dysmorphic face were the two other common clinical features in all FUT8-CDG, SLC35C1-CDG

(except one case), and GFUS-CDG patients, but it is notable that none of the FCSK-CDG patients presented with this clinical picture; on the other hand, seizure/epilepsy has been reported in almost all of the patients but not the GFUS-CDG patient (Hullen et al., 2021). These findings are compatible with "distinct GDP-fucose sources and different fucosylation reactions" as defective fucosylation of salvage and the de novo pathways, respectively due to *FCSK* and *GFUS* mutations, may result in different manifestations. However, the number of diagnosed patients is too low to conclude. POFUT1-CDG, the only CDG with defective fucosylation that has been reported with autosomal dominant inheritance, manifests a completely different clinical presentation with just skin involvement, known as Dowling-Degos Disease-2. It is attributed to the haploinsufficiency effect of POFUT1 on the role of the Notch signaling pathway in controlling proliferation and differentiation of melanocytes and keratinocytes (Li et al., 2013).

It is quite certain that CDGs with defective fucosylation are not as rare as it is thought to be. They cannot be detected by the transferrin glycosylation status-based standard screening method for CDG, due to very low amounts of fucose in transferrin; without the use of newer screening technology based on whole plasma proteins-derived N-glycans mass spectrometry and more importantly next-generation sequencing, many patients passed away without diagnosis. (Hullen et al., 2021; Lefeber et al., 2011) It is notable that four of the five known types of fucosylation defects have been discovered quite recently, (Hullen et al., 2021) and it cannot be ignored that more cases will be identified after describing a disorder. Given FCSK-CDG specifically, it is noteworthy that three of the five reported patients have Middle Eastern ancestry, (Al Tuwaijri et al., 2023; Manoochehri et al., 2022; Ng, Rosenfeld, et al., 2018; Ozgun & Sahin, 2022) which is well explainable by the recessive inheritance nature of this disease and the high rate of consanguineous marriage in some nations in the Middle East and Western Asia (Piedade et al., 2022). Since these are generally less-developed countries, a high underdiagnosis of FCSK-CDG and other fucosylation-related CDGs with autosomal recessive inheritance is expected.

5 | CONCLUSION

The present study utilized a CRISPR-generated cell model expanding insight into the molecular mechanism of this disorder. Our data indicated the significant effect of salvage pathway on EGF-like repeats O-fucosylation by a significant decrease in *NOTCH3* gene expression; it also suggests a probable preferential contribution of salvage pathway-originated GDP-fucose to secretory glycoproteins. Certainly, further studies are required to be conducted to reveal the underlying mechanisms of the disease.

AUTHOR CONTRIBUTIONS

Maryam Fazelzadeh Haghighi: conceptualization, methodology, investigation, writing—original draft. Hossein Jafari Khamirani: conceptualization, investigation. Jafar Fallahi: methodology. Ali Arabi Monfared: methodology. Korosh Ashrafi Dehkordi: conceptualization, validation, funding acquisition, writing—review and editing. Seyed Mohammad Bagher Tabei: conceptualization, supervision, writing- review and editing. All contributing authors approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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