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REVIEW

Suppressor tRNA in gene therapy

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Suppressor tRNAs are engineered or naturally occurring transfer RNA molecules that have shown promise in gene therapy for diseases caused by nonsense mutations, which result in premature termination codons (PTCs) in coding sequence, leading to truncated, often non-functional proteins. Suppressor tRNAs can recognize and pair with these PTCs, allowing the ribosome to continue translation and produce a full-length protein. This review introduces the mechanism and development of suppressor tRNAs, compares suppressor tRNAs with other readthrough therapies, discusses their potential for clinical therapy, limitations, and obstacles. We also summarize the applications of suppressor tRNAs in both *in vitro* and *in vivo*, offering new insights into the research and treatment of nonsense mutation diseases.

suppressor tRNA | PTC | nonsense mutation | readthrough | gene therapy

Introduction

Genetic disorders encompass a variety of diseases caused by genetic anomalies, whether inherited or acquired, leading to different levels of dysfunction in various human body parts. They are classified into several categories according to the genetic materials involved: single-gene disorders, multifactorial disorders, chromosomal disorders, and mitochondrial disorders. As hypothesized by the central dogma, DNA mutations may cause transcript inaccuracies, leading to abnormal protein synthesis. These abnormal or dysfunctional proteins ultimately cause moderate to severe symptoms of genetic disease. Among all pathogenic genetic mutations, nonsense mutation accounts for about 11% in human inherited diseases (Mort et al., 2008).

Nonsense mutation is defined as single-nucleotide substitution in a gene's in-frame coding sequence that results in a premature termination codon (PTC), which causes the translation to terminate too early. The shortened protein is often non-functional or only partially functional. Most of these genetic diseases are defined as rare diseases, lacking sufficient attention and financial support for the development of new treatment strategies.

Stop codon readthrough, which allows an amino acid to be incorporated into the premature stop codon, enabling the synthesis of full-length proteins, is a promising therapeutic approach for suppressing nonsense mutations.

Readthrough therapies have emerged and achieved exciting therapeutic effects in recent years, including readthrough drugs, inhibition of nonsense-mediated mRNA decay (NMD), PTC pseudouridylation, gene editing and suppressor tRNAs. The commonly used readthrough drugs such as aminoglycosides, traditionally known for their use as antibiotics, can impact the prokaryotic protein translation. Another well-known readthrough drug is PTC124 (also known as Ataluren). PTC124 is an oxadiazole compound that can readthrough PTCs by a similar mechanism with higher efficiency and minimal adverse effect compared to aminoglycosides (Welch et al., 2007). The European Union (EU) has given conditional approval for its use in treating Duchenne muscular dystrophy (DMD).

Among these readthrough strategies, suppressor tRNAs are engineering or naturally occurring transfer RNA molecules designed to recognize and pair with these premature stop codons, allowing the ribosome to continue translation and produce a full-length protein (Figure 1). Several research works have shown the potential of suppressor tRNAs in gene therapy for treating diseases resulting from nonsense mutations.

This review aims to compare suppressor tRNA and other translational readthrough therapies, explore the prospects of suppressor tRNAs for clinical therapy, as well as the current challenges and constraints that exist with suppressor tRNA therapy, and offer new insights for the research and treatment of nonsense mutation diseases. While other aspects of tRNA therapy also played substantial roles in the advancement of gene and cell therapy, their discussion is beyond the scope of this article, and we refer readers to relevant review articles on these topics (Coller and Ignatova, 2024).

Development of suppressor tRNAs

Transfer RNA (tRNA) is a type of RNA molecule carrying amino acids to the ribosome, assembled into a growing polypeptide chain during translation. Each specific amino acid is covalently bonded to the 3′ end of the corresponding tRNA with the catalysis of aminoacyl-tRNA synthetase (aaRS), creating a

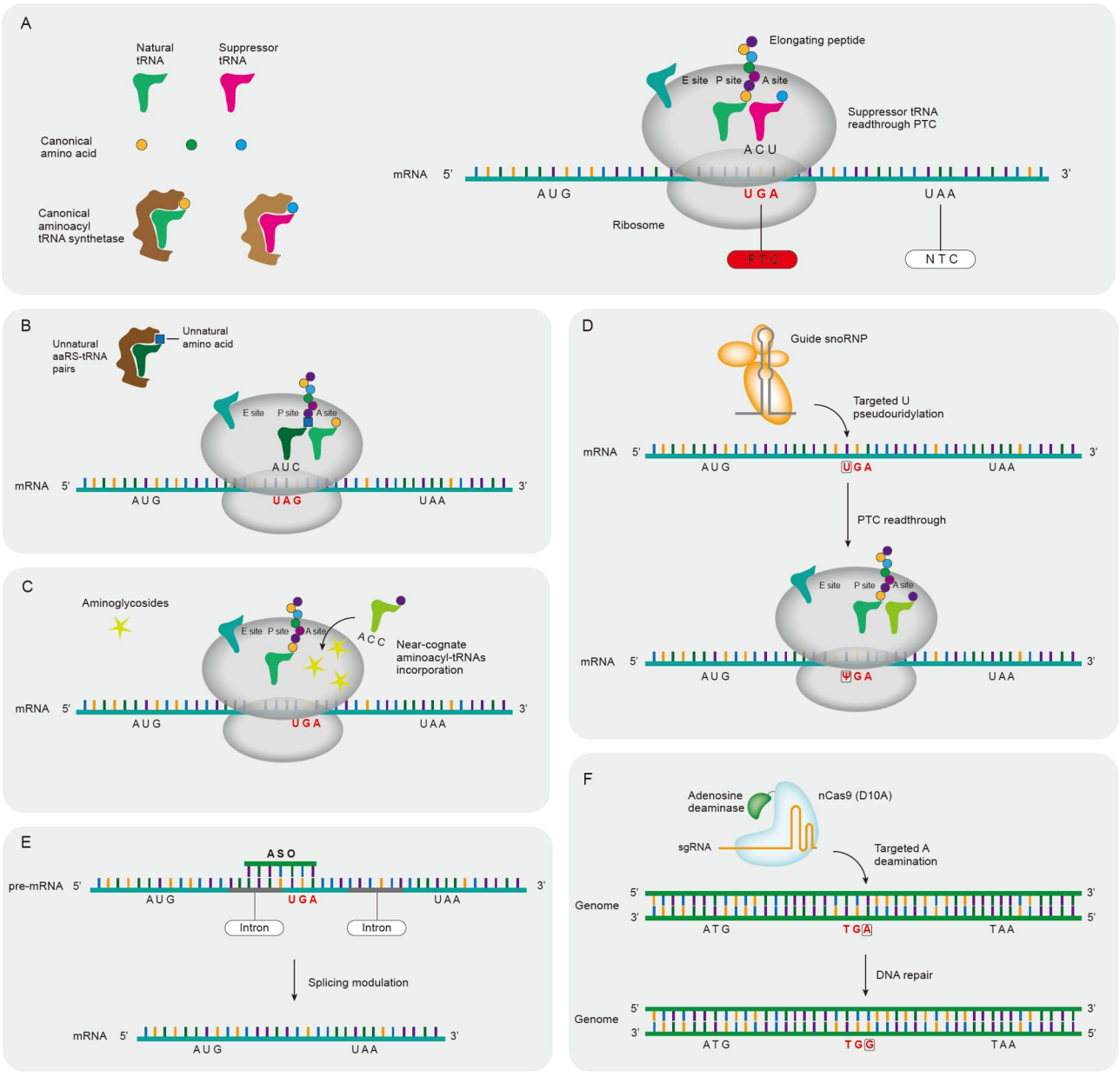

Figure 1. Mechanism of PTC readthrough strategies. A, Suppressor tRNA readthrough strategy. Natural tRNAs with engineered anticodons are charged with canonical amino acids to enable readthrough of the premature termination codon (PTC). B, Unnatural aaRS-tRNA pairs readthrough strategy. Unnatural aaRS-tRNA pairs facilitate readthrough of the PTCs by incorporation of unnatural amino acids. C, Aminoglycosides readthrough strategy. Aminoglycosides disrupt the ribosome decoding center, leading to misincorporation of the near-cognate aminoacyl-tRNA and subsequent readthrough of the PTCs. D, Pseudouridylation of PTCs strategy. Targeted pseudouridylation of uridine in PTCs suppresses PTC-induced translation termination. E, Antisense oligonucleotides (ASO) strategy. ASOs can be designed to modulate RNA splicing, resulting in the PTC-containing exon skipping. F, Gene editing strategy. Adenine base editors (ABEs) convert targeted A·T base pairs to G·C base pairs, thereby altering PTCs to normal coding sequences.

charged tRNA (also named aminoacyl-tRNA). Then the charged tRNA delivers the amino acid to the ribosome A site. It is commonly believed that tRNA is just a tool for transporting amino acids until the first discovery of suppressor tRNA. Suppressor tRNA is a tRNA derivative whose anticodon decodes one of three termination codons (Engelhardt et al., 1965). This modification enables the insertion of amino acids at termination codons, effectively bypassing the translation termination process.

In 1962, Benzer and Champe discussed two specific mutations termed 'nonsense mutation' and 'suppressor mutation'. They found that 'nonsense mutations' in the *r*II region of phage T4 lead to a disruption in the normal translation of genetic information into functional proteins. On the other hand, 'suppressor mutations' in the bacterium can counteract the effect of these nonsense mutations and allow the previously non-functional sequence to code for an amino acid again (Benzer and Champe, 1962). This indicated that suppressor mutations could add new meaningful coding units in the genetic code, and the nonsense mutation suppression occurred at the stage of mRNA being translated into protein. However, it was still unclear which components of protein synthesis are responsible for this suppression. Till 1965, Engelhardt et al. employed a strain of *Escherichia*

coli with the *su+* gene and phage f2 with a nonsense mutation, determining the coat protein to identify which components of protein synthesis are responsible for the suppression. The study demonstrated that suppressor RNA (sRNA, now known as suppressor tRNA) led to the insertion of the amino acid serine into the phage coat protein. While in the *su-* case, the production of coat protein fragments terminated at the same position (Engelhardt et al., 1965). This study provided new insight into the mechanism of *in vitro* suppression of nonsense mutation and the role of sRNA in this process. Besides, Gesteland et al. revealed that the amber mutations in T4 lysozyme could be suppressed by serine, tyrosine, and glutamine sRNA species, producing active enzymes (Gesteland et al., 1967)(Figure 2).

In 1979, Kohil et al. found that opal (UGA) suppressor tRNA purified from *Schizosaccharomyces pombe* could be aminoacylated with serine, indicating that it inserted serine in response to the UGA termination codon in rabbit globin mRNA (Kohli et al., 1979). This study was crucial for understanding the suppression of nonsense mutations in eukaryotes. In 1980, Bienz et al. utilized the *Xenopus laevis* oocyte as a sensitive *in vivo* screening system for amber (UAG), ochre (UAA), and opal eukaryotic nonsense suppressor tRNAs. They found that all three types of suppressor tRNAs could induce readthrough (Bienz et al., 1980). This research contributed significantly to understanding the mechanisms of nonsense suppression in eukaryotes, offering a practical and reliable system for studying suppressor tRNAs in a living cell. Similar readthrough phenomena have also been discovered in viruses and plants (Barciszewski et al., 1985; Bienz and Kubli, 1981). In 1982, Hudziak et al. constructed a single amber suppressor mutant of the *Xenopus laevis* $tRNA^{Tyr}$ gene and microinjected it into mammalian cell lines carrying multiple nonsense mutation genes. They found that the products of the suppressed amber mutant genes could be selectively expressed in mammalian cells and restore the gene function (Hudziak et al., 1982). This confirmed the functionality of suppressor tRNA in mammalian cells, had significant implications for genetic engineering, and opened avenues for further therapeutic strategies based on tRNA-mediated suppression. In 1987, Ho and Kan presented a method to quantify the tRNA aminoacylation *in vivo* in mammalian cells. It was found that the suppressor $tRNA^{Tyr}$ was fully aminoacylated, while suppressor $tRNA^{Lys}$ and $tRNA^{Gln}$ were aminoacylated at 40-50% and 80%, respectively (Ho and Kan, 1987). They provided valuable insights into the functionality of engineered suppressor tRNAs in eukaryotic cells and the importance of tRNA aminoacylation in the context of genetic mutations and disease mechanisms. Later, Kuchino et al. found that in mice infected with Moloney Murine Leukemia Virus (Mo-MuLV), the content of the UGA suppressor glutamine tRNA in the liver significantly increased (Kuchino et al., 1987). This was the first demonstration that in mammals, the amount of natural suppressor glutamine tRNA increased due to infection with viruses or changes in tumor cells.

With the development of genetic engineering technology, researchers have delved deeper into the mechanisms. In 1990, Kleina et al. used synthetic oligonucleotides to construct 17 types of amber suppressor tRNA genes originating from *Escherichia coli*. They found that different synthetic suppressor tRNAs showed significant differences in suppression efficiency. Modifications such as single-base changes could increase the efficiency of suppressor tRNAs (Kleina et al., 1990), making this study significant for understanding and applying suppressor tRNAs. In 2000, the first *in vivo* study of suppressor tRNA was conducted, where suppressor tRNAs were injected once into the hearts of transgenic mice expressing the chloramphenicol acetyltransferase (CAT) gene with a UAA nonsense mutation, resulting in partial restoration of CAT activity in about 10% of muscle fibers (Buvoli et al., 2000). In 2013, research showed that the expression of amber suppressor tRNAGln UAG in mammalian cells could be enhanced using the U6 promoter (Koukuntla et al., 2013). This demonstrated the possibility of enhancing suppressor tRNA expression through genetic engineering, providing a new strategy for gene therapy of genetic diseases by suppressor tRNAs. In 2019, Lueck et al. developed a high-throughput screening system to identify anticodon-engineered transfer RNAs (ACE-tRNAs) that could effectively suppress premature termination codons (Lueck et al., 2019). This showed the efficacy and safety of ACE-tRNAs under various *in vivo* and *in vitro* conditions, facilitating possibilities for future gene therapy of genetic diseases.

Frameshift suppressor tRNAs, another type of suppressor tRNAs, were first characterized in *Salmonella typhimurium* in 1973 (Riddle and Carbon, 1973). It restored in-frame decoding of the mRNA transcript containing a +1 frameshift mutation. A frameshift mutation occurs when adding or deleting one or several base pairs in a gene sequence disrupts the normal reading frame during protein synthesis, resulting in contiguous, improper amino acids (Roth, 1974). The additional structural and modifications in the sequence of frameshift tRNAs enable them to stabilize the ribosome in a conformation and promote decoding 4 nucleotides with a single tRNA rather than standard 3 nucleotides in other prokaryotic models (Hong et al., 2018; Maehigashi et al., 2014). However, the competition between frameshift suppressor tRNAs and natural tRNAs is stronger than suppressor tRNAs, which limits further study of frameshift suppressor tRNAs for therapeutic applications (Rodriguez et al., 2006).

Nonsense-associated diseases and therapies

Suppressor tRNA therapy in vivo

Several studies have demonstrated that tRNAs can potentially be used for human somatic gene therapy to treat diseases. In 1982, a functional human suppressor tRNA was engineered to suppress a UAG mutation in the β-globin gene, the causative gene for β-thalassemia, leading to successful β-globin restoration (Temple et al., 1982). Additionally, a human arginine UGA suppressor tRNA restored DNA repair in xeroderma pigmentosum cells (Panchal et al., 1999). Similarly, Kiselev et al. injected the plasmid expressing a UAA suppressor tRNA directly into the *mdx* DMD mouse quadriceps muscle (Kiselev et al., 2002). Approximately 2.5% of muscle fibers showed evidence of full-length dystrophin one week post injection.

Adeno-associated virus (AAV) vectors and lipid nanoparticle (LNP) are currently the leading viral and non-viral delivery systems for gene therapy (Cullis and Hope, 2017; Wang et al., 2019). In 2009, Koukuntla delivered suppressor tRNAGln UAG genes via AAV2 system into SEGHP mice carrying a nonsense-mutant GFP gene by tail vein injection (Koukuntla, 2009). Full-length GFP protein was restored successfully in the mice, which enlightened the systemic therapeutic application. Katrekar and Mali injected a designed AAV8 carrying the serine suppressor

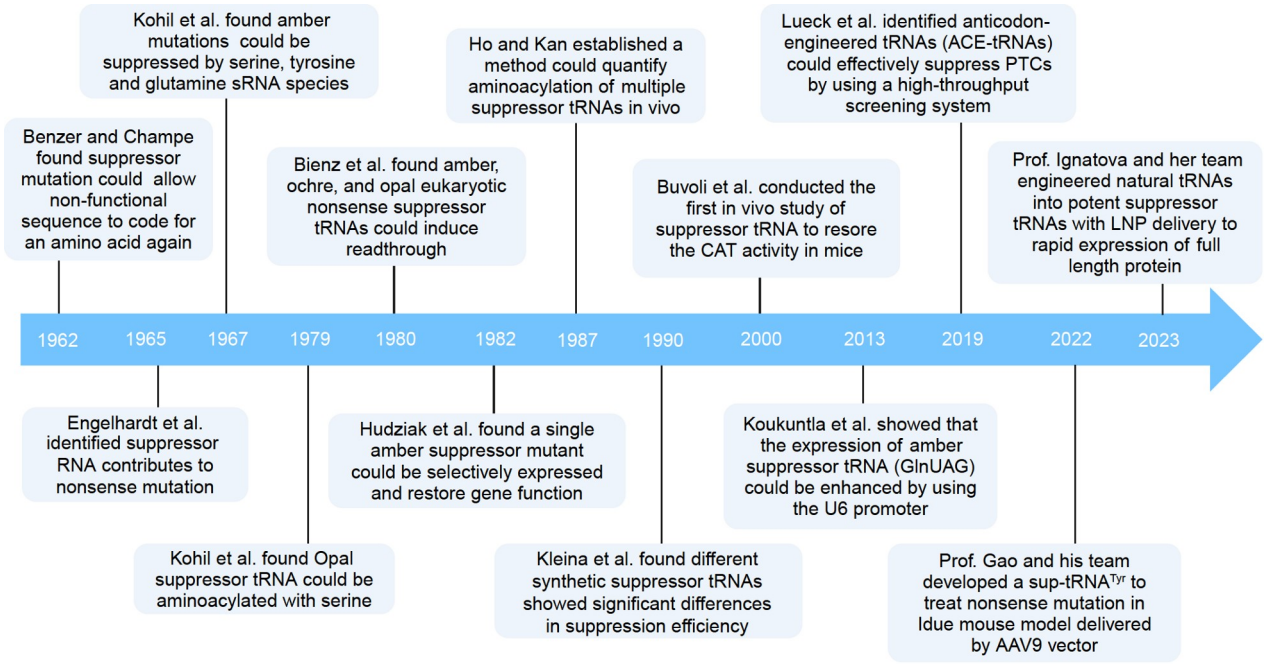

Figure 2. The development of suppressor tRNAs. A timeline is pictured showing selected milestones in the suppressor tRNA development. Since the first report on the discovery of 'nonsense mutation' and 'suppressor mutation' in 1962 (Benzer and Champe, 1962), the next years of basic biology researches gradually demonstrate the mechanism of the suppression of nonsense mutation by suppressor tRNAs (Engelhardt et al., 1965). It was found that all three types of stop codons in eukaryotes could be read through in 1980 (Bienz et al., 1980). Similar results were also reported in mammalian cells in 1982 (Hudziak et al., 1982). The first *in vivo* suppressor tRNA research by topical administration was conducted in 2000 (Buvoli et al., 2000). Two representative studies about systematic delivery of suppressor tRNA *in vivo* via Adeno-associated virus (AAV) and lipid nanoparticle (LNP) to treat nonsense-related diseases were reported in 2022 and 2023 (Albers et al., 2023; Wang et al., 2022). (Created with BioRender.com).

tRNA into the tibialis anterior or gastrocnemius of *mdx* DMD mice (Katrekar and Mali, 2017). Significantly improved restoration of dystrophin expression was observed after 8 weeks.

Recently, two representative works about the systematic delivery of suppressor tRNA *in vivo* to treat nonsense-related diseases have emerged. In 2022, Prof. Gao and his team showcased the potential of AAV-delivered nonsense suppressor tRNAs that function on premature termination codons suppression to rescue disease-causing nonsense mutations and reinstate gene function through natural regulation (Wang et al., 2022). Ten weeks after injection, IDUA activity was restored to 2.5% in serum, 9.5% in liver, and 27% in the heart of normal levels, leading to a significant reduction of GAG accumulation, and the effects lasted for more than 6 months after a single injection. In 2023, Prof. Ignatova and her team utilized a strategy for altering natural tRNAs into potent suppressor tRNAs by individually fine-tuning their sequences to suit the physicochemical properties of the amino acids they carried (Albers et al., 2023). Besides, they leveraged a synthetic LNP system to encapsulate suppressor tRNA and delivered the LNP-sup-tRNA via intravenous injection. After 6 h after administration, up to 66% of readthrough was detected, suggesting a rapid and efficient production of functional protein.

As a therapeutic approach for nonsense-associated diseases, suppressor tRNAs possess kinds of advantages: (1) one therapeutic formulation can be applied to a diverse array of distinct clinical conditions with the same nonsense mutation; (2) the suppressor tRNAs are short and can be easily packaged for flexible delivery, such as by AAV or LNP; (3) suppressor tRNA specifically utilizes the endogenous impacted transcripts, avoiding the cytotoxicity resulting from exogenous protein overexpression or off-target expression. For example, in MECP2-knockout mice (mouse model of Rett Syndrome), moderate overexpression of MECP2 enhances motor coordination and attenuates anxiety and situation-dependent fear (Collins et al., 2004), whereas higher levels (2 to 4 times) of MECP2 expression in neurons results in tremor and motor dysfunction in mice (Luikenhuis et al., 2004). This makes suppressor tRNA well-suited for treating diseases where gene expression must be regulated within a narrow physiological range.

Why are suppressor tRNAs more sensitive to PTCs? Amrani et al. suggested that the ribosome stayed longer at normal termination codons than at PTCs, indicating that the efficiency of translation termination at normal termination codons is higher than at PTCs (Amrani et al., 2004). This creates conditions for the read-through of suppressor tRNAs. Research by Hoshino et al. showed that eRF3 interacts with the poly(A)-binding protein (PABP) at the 3′ end of mRNA to enhance the efficiency of translation termination (Hoshino et al., 1999). At the normal termination codon, eRF3 is closer to PABP, which aids the interaction; but at PTC, it is farther from PABP, making the interaction weaker. In actual practice, the abundance of PABP, the relative length of the 3′UTR, and the nucleotide sequence around the termination codon also affect read-through (Keeling et al., 2012).

The incorporation of unnatural amino acids (UAAS)

The combination of these 20 amino acids and the interactions between their side chains determines the diversity of protein functions. However, there is a wide array of unnatural amino acids with structural and functional diversity far exceeding that

of natural amino acids. Professor Peter G. Schultz and his research team were the first to establish the technique of genetic codon expansion (Xie and Schultz, 2006). This technique allows researchers to site-specifically introduce over 200 unnatural amino acids containing various functional side chain groups into target proteins with the aid of special codons such as UAG and UAGA, enhancing the biochemical properties of proteins or peptides from single-celled to multi-celled organisms, and from lower to higher species (Bessa-Neto et al., 2021; Robertson et al., 2021; Zhao et al., 2021). However, there have been few reports on using unnatural aaRS-tRNA pairs to restore endogenous protein expression and treat nonsense mutation diseases.

In 2021, Dr. Xia and her group discussed restoring dystrophin expression in mice by suppressing a nonsense mutation by incorporating unnatural amino acids (Shi et al., 2022). By delivery of engineered aaRS-tRNA pairs and unnatural amino acids via AAV, the study demonstrated partial restoration of dystrophin expression in myoblasts derived from mdx mice and a DMD patient, as well as substantial restoration of muscle function up to 8 weeks in mouse models of DMD. This approach offers a new strategy for treating DMD and other similar diseases caused by nonsense mutations.

Aminoglycosides

Aminoglycosides are a class of antibiotics consisting of a 2-deoxystreptamine ring linked to multiple amino sugars (Lombardi et al., 2020). They bind tightly to the ribosomal decoding center, leading to misincorporation of the near-cognate aminoacyl-tRNAs and extensive codon misreading (Recht, 1999). However, in eukaryotic cells, the affinity of aminoglycosides for the eukaryotic decoding center was significantly reduced due to differences at two key nucleotides of ribosomal RNA sequence (Lynch and Puglisi, 2001; Recht, 1999). Nevertheless, the impact of aminoglycosides on the eukaryotic translation elements is sufficient enough to lead to the misincorporation of near-cognate aminoacyl-tRNAs at the A site and induce readthrough at PTCs (Dabrowski et al., 2018; Lombardi et al., 2020).

Dozens of *in vitro* and *in vivo* studies have reported that aminoglycosides could suppress translation terminations at PTCs and restore the function of full-length proteins. Gentamicin is the most commonly used aminoglycoside for nonsense suppression studies. About half of cystic fibrosis (CF) patients successfully restored the functional Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein in nasal epithelia 1-2 weeks after intravenous or nasal droplet administration of gentamicin (Clancy et al., 2001; Wilschanski et al., 2003). Besides, Barton-Davis et al. demonstrated that gentamicin can promote PTC readthrough in the *mdx* mice and generate a full-length dystrophin protein (Barton-Davis et al., 1999). Similarly, an increase in dystrophin protein in muscle biopsies and a decrease in serum creatine kinase were observed in part of DMD patients treated with gentamicin (Malik et al., 2010; Politano et al., 2003; Wagner et al., 2001). Gentamicin applied topically emerges as a highly promising treatment for genodermatoses caused by nonsense mutations. Clinical research has been conducted on the use of topical gentamicin for treating four distinct genodermatoses, including Nagashima-type palmoplantar keratosis (NPPK) (Li et al., 2021; Ohguchi et al., 2018), epidermolysis bullosa (Kwong et al., 2020; Li et al., 2020; Woodley et al., 2017), Hailey-Hailey disease (HHD) (Kellermayer et al., 2006), and hereditary hypotrichosis simplex of the scalp (HSS) (Peled et al., 2020), and patients participated in the treatment experienced benefits without significant side effects. But no clear clinical efficiency was achieved. However, it seems that gentamicin is not effective enough to have therapeutic benefits for patients with hemophilia or McArdle disease (Schroers et al., 2006; Shiozuka et al., 2010).

These studies indicate that only a fraction of patients with nonsense mutations can restore a significant amount of functional proteins by administration of gentamicin. In addition, due to the toxic side effects of traditional aminoglycosides, such as hearing loss and kidney damage, it is unlikely to use aminoglycosides for long-term treatment (Ali et al., 2011; Huth et al., 2011).

PTC124 (Ataluren)

PTC124, also known as Ataluren, is an oxadiazole compound discovered by PTC Therapeutics that suppresses termination at PTCs in mammalian cells (Welch et al., 2007). Several investigations *in vitro* and *in vivo* have produced positive results that PTC124 suppresses nonsense mutations and restores deficient protein function associated with numerous disorders, such as DMD (Welch et al., 2007), CF (Gonzalez-Hilarion et al., 2012), mucopolysaccharidosis type I (MPS I) (Peltz et al., 2013), Usher syndrome (Goldmann et al., 2012; Goldmann et al., 2011), propionic acidemia (Sánchez-Alcudia et al., 2012), carnitine palmitoyltransferase 1A deficiency (Tan et al., 2011), methylmalonic aciduria (Buck et al., 2012) and so on. To date, studies have reported on the activity of Ataluren in promoting the readthrough of PTCs in almost 40 preclinical models of genetic disorders caused by nonsense mutations. In mouse models of DMD and CF, PTC124 effectively suppressed nonsense mutations, restoring about 20% of the functional protein (Du et al., 2008; Welch et al., 2007). Phase I clinical studies showed promising safety and efficacy, driving further phase II/III clinical trials. In 2014, the European Union conditionally approved PTC124 for use in patients over 5 years old (Ryan, 2014). By 2018, the indication had been expanded to include those aged between 2 and 5 years old who can walk (Michorowska, 2021). PTC124 is also conditionally approved for the treatment of nonsense mutation DMD in Great Britain, Russia, the Republic of Korea, Brazil, and other countries (McDonald et al., 2022). However, PTC124 is still an investigational drug in the United States because the FDA has rejected PTC124's application for DMD via necessitating confirmatory clinical trials (Michorowska, 2021). The recent update to the EU label now endorses the use of Ataluren in patients who have lost the ability to walk while undergoing treatment.

However, several studies have discovered that PTC124 and its analogs are powerful inhibitors of Firefly Luciferase (FLuc), enhancing cellular FLuc activity through posttranslational stabilization rather than inducing readthrough of PTCs. This implies that the observed activity of ataluren might be due to an off-target effect on the FLuc reporter, rather than its intended action on PTC readthrough (Auld et al., 2010; Auld et al., 2009). Furthermore, PTC124 was unable to read through PTCs in several disease models including peroxisome biogenesis disorders, frontotemporal dementia, monogenetic obesity, and long-QT syndrome (Bolze et al., 2017; Dranchak et al., 2011; Harmer et al., 2012; Kuang et al., 2020).

Pseudouridylation

Pseudouridylation, one of the most common single-nucleotide modifications of functional RNAs, is the isomerization of the ribonucleoside uridine to the 5′-ribosyl isomer pseudouridine (Ψ). Compared with uridine, ψ has an extra hydrogen-bond donor at its N1 position and it is more polar, making it capable of profoundly influencing the properties of the RNA molecule and the cellular process (Ejby et al., 2007; Tomita, 1999). Box H/ACA guide RNAs, a group of noncoding RNAs, are responsible for recognizing the substrate RNA by base-pairing interactions and directing site-specific pseudouridylation (Ni et al., 1997). By constructing artificial guide RNAs, researchers found that site-specific modification can be introduced post-transcriptionally into a variety of RNA molecules (Ge et al., 2010; Wu et al., 2011; Zhao and Yu, 2008).

A uridine residue consists in the first position of all three nonsense codons, making it possible to introduce pseudouridylation into a PTC to reach nonsense suppression. Studies have demonstrated that pseudouridylation of stop codons resulted in readthrough and full-length protein synthesis both *in vitro* and *in vivo* (Huang et al., 2012; Karijolich and Yu, 2011). Adachi and Yu found that this pseudouridine (Ψ)-mediated readthrough is not dependent on the mRNA′s sequence context, indicating its potential as a broad-spectrum strategy for addressing genetic diseases caused by nonsense mutations (Adachi and Yu, 2020). Adachi et al. applied RNA-guided RNA pseudouridylation to disease model cell lines of DMD and MPS I, showing effective nonsense suppression and functional restored proteins (Adachi et al., 2023). Song et al. reported that a programmable RNA base editor named RESTART, which utilizes an engineered guide snoRNA (gsnoRNA) and the endogenous H/ACA box snoRNP machinery to achieve precise pseudouridylation, enables PTC readthrough in mammalian cells (Song et al., 2023).

Nonetheless, site-directed pseudouridylation as a readthrough therapy for nonsense mutation related diseases still has some obstacles. The insertion of Ψ might change RNA structure or even protein property. Future studies are desired to overcome the off-target effects, and the safety of pseudouridylation awaits further investigation in animal models.

ASO

Antisense oligonucleotides (ASOs) are short, synthetic strands of nucleic acids designed to specifically bind to and interfere with the mRNA of target genes (Askari and McDonnell, 1996). Exon skipping ASOs are a therapeutic approach used in the treatment of nonsense mutation diseases. These ASOs are designed to bind to specific sequences in the mRNA, causing the skipping of an exon that contains the mutation during the mRNA processing, producing a truncated but potentially functional protein. Besides, Nomakuchi et al. used ASOs to selectively inhibit NMD by targeting and blocking the sites where exon-junction complexes (EJCs) are deposited on mRNA downstream of a premature termination codon. This approach prevents the assembly of NMD machinery on the targeted mRNA, allowing it to escape degradation (Nomakuchi et al., 2016). Currently, ASOs have been proven to effectively bypass protein-truncating mutations in patient cell lines of CF and the mouse model of DMD.

There are still some challenges associated with ASO therapy, including the degradation of oligonucleotides by DNases, which are widespread in the body, the requirement for large doses to achieve therapeutic effects, difficulty in targeting specific cells, and the necessity for parenteral (non-oral) administration. Additionally, oligonucleotides have a transient half-life in plasma, making it essential to chemically modify them to enhance their stability and reduce degradation.

NMD inhibition

NMD is the RNA surveillance mechanism that detects and degrades mRNA molecules containing PTCs (Nicholson et al., 2010; Sato and Singer, 2021). Based on its role in regulating the abundance of transcripts containing PTCs, NMD represents a potential therapeutic target for diseases caused by PTCs. NMD inhibition therapy is an emerging therapeutic strategy for treating these diseases.

It is proved that depletion of SMG1 or SMG6, key factors in NMD-associated pathways (Kurosaki et al., 2019; Schweingruber et al., 2013), leads to significant increases in truncated CFTR protein levels and function were observed in cell lines (Sanderlin et al., 2022). Besides, variable NMD efficiency among patients also influences the effectiveness of nonsense suppression therapy. Patients with higher levels of nonsense transcripts (due to less efficient NMD) responded better to other readthrough therapies than those with lower transcript levels (Kerem et al., 2008; Linde et al., 2007). Researchers also found that combining readthrough drugs with NMD inhibitors would synergistically affect the levels of nonsense-containing mRNAs and may increase the full-length protein levels (Amar-Schwartz et al., 2023; McHugh et al., 2020).

NMD inhibition offers a new approach to treating diseases caused by premature termination codons, but it requires careful consideration due to potential side effects. NMD targets transcripts with PTCs resulting from mutations and regulates a significant portion of the mammalian transcriptome, including various types of natural mRNA substrates (Mendell et al., 2004). Additionally, NMD factors like UPF1, UPF2, and SMG-1 are crucial for embryonic development, with their knockout leading to embryonic lethality in mice (McIlwain et al., 2010; Medghalchi, 2001; Weischenfeldt et al., 2008).

Gene editing

The advent of genome editing technologies, especially clustered regularly interspaced short palindromic repeat (CRISPR) gene editing, has opened an entirely new field with numerous opportunities for potential nonsense suppression therapies. The utilization of CRISPR-Cas9 system to correct disease-associated nonsense mutation shows significant restoration of protein function in mouse models of DMD, CF, Leber congenital amaurosis (LCA) and familial hypercholesterolemia (Afanasyeva et al., 2023; Jo et al., 2019; Nelson et al., 2016; Santos et al., 2022; Tabebordbar et al., 2016; Zhao et al., 2020). Besides, CRISPR-Cpf1 (also known as CRISPR-Cas12a) was employed to correct DMD mutations in patient-derived induced pluripotent stem cells (iPSCs) and *mdx* mice (Zhang et al., 2017).

Adenine base editors (ABEs), derived from the CRISPR-Cas system, enable the direct, irreversible transition of adenine(A) to guanine(G) in the DNA without creating double-strand breaks (Gaudelli et al., 2017). A few groups have reported ABEs restored the open reading frame in PTCs successfully in patient fibroblasts

of recessive dystrophic epidermolysis bullosa (RDEB), organoid of CF, and mouse models of DMD and LCA (Geurts et al., 2020; Ryu et al., 2018; Sheriff et al., 2022; Suh et al., 2021). Lee et al. established an ABE-mediated readthrough method, named CRISPR-pass, to bypass PTCs by converting adenine to guanine or thymine to cytosine (Lee et al., 2019). CRISPR-pass efficiently rescued functional protein expression in knockin (KI) Hela cell lines with six different types of PTCs and in patient fibroblasts with *xeroderma pigmentosum, complementation group C* (*XPC*) gene mutation. Yuan et al. explored the innovative use of a CRISPR-guided cytidine deaminase, known as targeted-AID mediated mutagenesis (TAM), for modulating various forms of mRNA splicing. They genetically restored the open reading frame and dystrophin function of a mutant DMD gene in patient-derived induced pluripotent stem cells (iPSCs) (Yuan et al., 2018).

Prime editing (PE) is a new CRISPR-based tool that can engineer precise genome edits without double-strand break or exogenous donor DNA template (Anzalone et al., 2019). She et al. integrated the optimized split PE into dual-AAV vectors to target the nonsense mutation of the *Rpe65* gene in *rd12* mice, a mouse model of LCA, showing restored RPE65 expression, rescued retinal and visual function, and preserved photoreceptors (She et al., 2023). Utilizing an optimized PE2 protein system, Xia et al. recovered testosterone production, restarted sexual development, rescued spermatogenesis, and produced fertile offspring in the nonsense-point-mutation mouse model of hereditary primary hypogonadism (HPH) (Xia et al., 2023).

As a key mechanism for reprogramming genetic information at the post-transcriptional level, RNA editing plays a complementary and perhaps even more nuanced role in the regulation of gene expression alongside genome editing. Adenosine-to-inosine (A-to-I) RNA editing is a common post-transcriptional modification catalyzed by adenosine deaminases acting on RNA (ADAR) enzymes (Melcher et al., 1996; Nishikura, 2010). For years, recruiting endogenous adenosine deaminases using exogenous guide RNAs to edit cellular RNAs has been a promising therapeutic strategy. Researchers engineered circular ADAR-recruiting guide RNAs and found that AAV-mediated delivery of circular ADAR-recruiting RNAs rectified the premature stop codon precisely and restored the functionality of IDUA enzyme in the mouse model of MPS I-Hurler syndrome (Katrekar et al., 2022; Yi et al., 2023). Li et al. demonstrate a mini-dCas13X–mediated RNA adenine base editing (mxABE) strategy rescued dystrophin expression and muscle function significantly via A-to-G editing in a genetically humanized mouse model of DMD (Li et al., 2023).

However, gene editing technologies have several limitations and challenges, including off-target effects, ethical issues, delivery method, efficiency, immune response, and so on. Still, the long-term safety and effects of gene editing are not yet fully understood.

We summarize and compare the nonsense suppression strategies in Table 1.

Indications of suppressor tRNA

The door to precision medicine is opening, but designing unique gene therapy strategies for each disease would be a prohibitively expensive project. Suppressor tRNAs can read through one of the three termination codons and ideally be charged with the same amino acid whose codon is mutated to PTC. Therefore, the one suppressor tRNA could have the potential to rescue different diseases caused by the same PTC. Therefore, suppressor tRNAs are suitable for diseases with a high frequency of nonsense mutations, as well as diseases caused by large genes with nonsense mutations, such as DMD. The following table summarizes the diseases that may be candidates for suppressor tRNA treatment, almost all of which are included in the two batches of rare disease lists announced in China (gov.cn/zhengce/zhengceku/2018-12/31/content_5435167.htm; gov.cn/zhengce/zhengceku/202309/content_6905273.htm) (Table 2).

Summary

Genetic diseases, resulting from abnormalities in the genome, have a wide range of impacts on human health. Nonsense mutations, in particular, disrupt protein synthesis and contribute to genetic diseases. Suppressor tRNA, an innovative therapeutic tool, can recognize and introduce the corresponding amino acids when the ribosomal translation encounters a PTC, allowing the production of full-length, functional proteins. The development and application of suppressor tRNA have evolved significantly since their discovery. Early studies in bacteria demonstrated their ability to override nonsense mutations, and further research in eukaryotes has solidified their potential in gene therapy (Barciszewski et al., 1985; Bienz et al., 1980; Engelhardt et al., 1965). *In vivo* studies, particularly in mouse models, have shown promising results, with suppressor tRNAs partially restoring protein function in diseases like DMD and MPS I (Katrekar and Mali, 2017; Wang et al., 2022).

Advancements in tRNA-based therapies

These promising preclinical studies have propelled biotechnology companies to start developing suppressor tRNA-based therapies for the treatment of nonsense-associated diseases, including CF, DMD, Rett syndrome, and Dravet syndrome. In November 2021, Alltran, a startup incubated by Flagship Pioneering, officially announced its launch, proclaiming itself as 'the world's first tRNA platform company' (Dolgin, 2022). Alltrna is not the only biotech company developing research on tRNA-based therapies. Before this, companies such as ReCode Therapeutics (recodetx.com), Shape Therapeutics (shapetx.com), hC Bioscience (hcbioscience.com), and Tevard Biosciences (tevard.com) have all conducted research and development in tRNA therapies (Anastassiadis and Köhrer, 2023). Professor Xia from Peking University founded QiXia Decode Therapeutics, focusing on using engineered tRNA–enzyme pairs to treat muscular dystrophy and cancer caused by nonsense mutations. By designing engineered tRNAs to carry the correct amino acids and introducing them at the site of mutation-generated stop codons, these companies focus on tRNA therapy that enables the continuation of protein translation, thereby achieving the goal of treating diseases.

Challenges and prospects

While suppressor tRNA therapy holds great promise for treating genetic diseases caused by nonsense mutations, several hurdles remain to overcome. The efficiency of PTC suppression by suppressor tRNAs depends on the surrounding sequence context

Table 1. Comparison of readthrough strategies in nonsense-associated diseases

Readthrough therapy strategies	Method	Advantage	Disadvantage	Comment
Suppressor tRNA therapy	Engineering or naturally occurring transfer RNA designed to recognize and pair with premature stop codons to produce a full-length protein.	One therapeutic formulation can be applied to a diverse array of distinct clinical conditions with the same PTC mutation, precisely incorporate the same amino acid in PTC site, easy to transfer and deliver.	Low charge efficiency with a specific amino acid by natural aminoacyl tRNA synthetase, cognate amino acid incorporation of PTC site, long expression may be toxic in normal stop codons.	The effect of suppressor tRNA on normal stop codons in vivo should be further investigated.
Unnatural amino acid incorporation system	Unnatural amino acid can recognize special codons such as UAG and UAGA to read through PTCs.	Over 200 non-natural amino acids containing various functional side chain groups can enhance the biochemical properties of proteins or peptides, the dose of administer can be control.	Unnatural amino acid may change the properties of target proteins.	Similar to suppressor tRNAs, still need more safety assessments.
Aminoglycosides	Aminoglycoside antibiotics typically bind to the A site of the 30S ribosomal subunit RNA, leading to misreading of genetic codons and readthrough of PTCs.	Easy to deliver orally or intravenously, the dose of administration can be controlled.	Codon and context dependent, long-term administration of aminoglycosides may result in renal damage and ototoxicity.	Because of the low efficiency and negative effects, clinical use of aminoglycoside antibiotics has been significantly limited.
PTC124	PTC124 targets the ribosome and promotes the insertion of near-cognate tRNA at nonsense coding sites.	PTC124 is more effective in the treatment of nonsense mutations than aminoglycosides drugs, most adverse post-treatment events are mild or moderate.	Inaccurate insertion of amino acid in PTC site leads to loss of function or misfold of target proteins. PTC124 can only treat a limited number of diseases caused by nonsense mutations.	Limited application in other PTC-induced disease models, screening other PTC124-like drugs may solve this issue.
Pseudouridylation	The Box H/ACA ribonucleoprotein (RNP) complex catalyzes the isomerization of uracil residues (U) to pseudouridine (Ψ), ψ has an extra hydrogen-bond donor at its N1 position, resulting in the binding of near-cognate amino acids.	Site-specific pseudouridylation of mRNA using guide RNA, readthrough can be achieved at multiple disease-causing gene loci without considering the effect on normal codons.	pseudouridylation only promotes mis-binding of near cognate amino acids, and targets sequence-specific pathogenic PTC, but cannot readthrough other pathogenic mutations caused by the same PTC.	Further studies are desired to investigate the consequence of off-target pseudouridylation, and the roles of DKC1 enzyme should be fully explored in cancers as well as other clinical cases.
Antisense oligonucleotides	By designing Antisense oligonucleotides, several disease-caused frameshift or nonsense mutations can be skipped at the mutated exon and produce a shorter but still partially functional protein.	ASOs allows for the selective inhibition of specific genes, a wide range of genetic targets can be targeted including protein-coding genes, non-coding RNAs, and splicing events, ASOs is easy to synthesize for therapeutic testing or treatment.	Easy to degrade, require large doses to achieve therapeutic effects, difficult in target-specific tissue, need frequent administration.	More studies are required to further improve the safety, tolerability and the administration frequency of ASOs.
Genome editing	Genome editing can precisely target the mutations in genome and correct it to wild type by using gene editing tools, such as base editing or prime editing.	Genome editing can precisely change the mutated DNA sequence, and permanently correct the mutation in genomic level, with no limitation on the mutation types or genomic loci.	Off-target effects, undesired by-products, immune response of the gene editing agents	The safety and purity of genome editing should be further improved, the delivery of gene editing components should be further optimized.

and the position of the PTC. By understanding and utilizing these context-dependent factors, it may be possible to design therapies that promote readthrough specifically at PTCs while minimizing readthrough at normal stop codons. For example, certain sequence motifs or secondary structures in the mRNA that follow the PTC could be targeted to facilitate sup-tRNA action (Escobar et al., 2023). Modifications in the anticodon of tRNAs may result in lower aminoacylation efficiency and inefficient nonsense suppression activity (Giege et al., 1998). Besides PTCs, suppressor tRNAs can also recognize normal termination codons (NTC), which may potentially produce abnormal protein resulting in deleterious effects. Engineering of certain stem-loop structures of sup-tRNA combined with high-throughput screening could selectively identify ideal variants with enhanced specificity for PTCs over normal termination codons, which could be one approach. This could be achieved by modifying the tRNA's anticodon or other regions to increase its affinity for the PTC while reducing its interaction with normal stop codons. Moreover, studies show that suppressor tRNA may incorporate missense amino acids while inducing readthrough (Wang et al., 2022). The usage of unnatural nucleotides for tRNA modification may be harmful and cause cytotoxicity (Swayze et al., 2007). Techniques like ribosome profiling, tRNA sequencing, and proteomics analysis may help to evaluate the safety (Ingolia et al., 2009; Zheng et al., 2015). Another hindrance to the development of suppressor tRNA therapy is the lack of experiments in big animals or non-human primates. Ultimately, delivery systems such as AAVs and LNP may trigger immune responses or cause cytotoxicity with high doses (Kedmi et al., 2010; Pupo et al., 2022). However, no tRNA therapies have been approved for clinical trials yet. With the rapid development of tRNA-related molecular biology, bioinformation, synthetic biology, and delivery systems, suppressor tRNA for gene therapy is entering a new era.

Table 2. Rare diseases that are potential targets for suppressor tRNA therapy

Disease	Mutate gene	cDNA(bp)	Incidence condition[a)]	Nonsense mutation/ pathogenic mutation[b)]	Ref.
Asphyxiating Thoracic Dystrophy	DYNC2H1	12945	Asphyxiating thoracic dystrophy affects an estimated 1 in 100,000 to 130,000 people.	27/253	Inserra et al., 2023
Carnitine Deficiency	SLC22A5	1746	Carnitine deficiency occurs in approximately 1 in 100,000 newborns worldwide. In Japan, this disorder affects 1 in every 40,000 newborns.	19/125	Lodewyckx et al., 2023
Congenital hyperinsulinism hypoglycemia	ABCC8	4812	Congenital hyperinsulinism affects approximately 1 in 50,000 newborns. This condition is more common in certain populations, affecting up to 1 in 2,500 newborns.	33/220	Dunne et al., 1997
Myotonia congenita	CLCN1	2967	Myotonia congenita is estimated to affect 1 in 100,000 people worldwide. This condition is more common in northern Scandinavia, where it occurs in approximately 1 in 10,000 people.	110/771	Papponen et al., 1999; Sun et al., 2001; Emery, 1991
Fabry Disease	GLA	1413	Fabry disease affects an estimated 1 in 1,000 to 9,000 people. Milder, late-onset forms of the disorder are probably more common than the classic, severe form.	75/777	Spada et al., 2006; Burton et al., 2017
Alagille Syndrome	JAG1 NOTCH2	3657 7416	The estimated prevalence of Alagille syndrome is 1 in 70,000 newborns.	247/1077 23/54	Mitchell et al., 2018
Choroideremia	CHM	1962	The prevalence of choroideremia is estimated to be 1 in 50,000 to 100,000 people. However, it is likely that this condition is underdiagnosed because of its similarities to other eye disorders. Choroideremia is thought to account for approximately 4 percent of all blindness.	289/934	Brambati et al., 2019
Laron syndrome	GHR	1938	Laron syndrome is a rare disorder. About 350 people have been diagnosed with the condition worldwide. The largest single group of affected individuals (about 100 people) lives in an area of southern Ecuador.	29/104	Werner, 2022
Osteogenesis Imperfecta	COL1A1	4395	Osteogenesis imperfecta affects approximately 1 in 10,000 to 20,000 people worldwide. An estimated 25,000 to 50,000 people in the United States have the condition.	257/3823	Pomerance, 1997
Glutaric acidemia type I	GCDH	1317	Glutaric acidemia type I occurs in approximately 1 in 100,000 individuals. It is much more common in the Amish community and in the Ojibwa population of Canada, where up to 1 in 300 newborns may be affected.	39/1099	Therrell Jr et al., 2014
Rett syndrome	MECP2	1497	This condition affects an estimated 1 in 9,000 to 10,000 females.	1269/3452	Bienvenu and Chelly, 2006

a) The incidence condition data were derived from MedlinePlus medical library. b) The nonsense mutation data were derived from Global Variome shared LOVD

Compliance and ethics

Y.Z. is a scientific co-founder of VecX Biomedicines. The other author(s) declare no conflict of interest.

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