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Review of the Pathological Mechanism and Development of Treatments for Retinitis Pigmentosa with *Mer Tyrosine Kinase* Mutations Using Patient-Derived Induced Pluripotent Stem Cells

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Abstract

Retinitis Pigmentosa (RP) is an incurable disease for which effective treatments are lacking. *Mer tyrosine kinase* (*MERTK*) is a causative gene of RP. Royal College of Surgeons rats with a *Mertk* mutation showed impaired phagocytosis of the photoreceptor outer segment by the Retinal Pigment Epithelium (RPE). Using RPE differentiated from induced Pluripotent Stem Cells (iPSC-RPE) of RP patients carrying *MERTK* mutations, recent studies have shown deterioration of phagocytosis in the iPSC-RPE. This review focused on the function of phagocytosis of iPSC-RPE cells derived from RP patients carrying *MERTK* mutations and discussed the possibility of developing treatments for this disease using this *in vitro* disease model.

Keywords: Retinitis pigmentosa • *Mer tyrosine kinase* • Induced pluripotent stem cells • Retinal pigment epithelium • Photoreceptor outer segment • Phagocytosis

Abbreviations

RP: Retinitis Pigmentosa; RPE: Retinal Pigment Epithelium; OS: Outer Segment; *MERTK*: *Mer tyrosine kinase*; TAM: Tyro3/Axl/Mer; POS: Photoreceptor Outer Segment; RCS: Royal College of Surgeons; iPSCs: Induced Pluripotent Stem Cells; iPSC-RPE: RPE cells differentiated from iPSCs; GAS6: Growth-Arrest-Specific 6; FITC: Fluorescein-5-isothiocyanate; MTK iPSC-RPE: Patient-derived iPSC-RPE; NOR iPSC-RPE: Healthy control-derived iPSC-RPE; PTC: Premature Termination Codon; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

Introduction

Retinitis Pigmentosa (RP) is an incurable disease that leads to blindness and affects approximately 1 in 4000 people [1]. This disease is characterized by progressive degeneration of photoreceptors and Retinal Pigment Epithelium (RPE) in the eye [1]. Photoreceptor cells have a disk-shaped membrane structure called the Outer Segment (OS). In the OS of photoreceptor cells, light perception occurs and the light stimulus is converted into an electrical signal [2]. RPE cells play an important role in preventing light scattering, phagocytosing the outer nodes of photoreceptor cells, and recycling substances necessary for light perception [2]. Most patients with RP follow the course of the disease with dark adaptation, night blindness, and loss of the mid-peripheral visual field. As the disease progresses, they lose peripheral visual field and finally lose central vision usually by the age of 60 [1], though there are large individual differences in progress. RP is diagnosed based on hearing of symptoms and family history and comprehensive ophthalmologic examinations. These include visual acuity measurements, slit-lamp biomicroscopy, ophthalmoscopy, fundus photography, Optical Coherence Tomography (OCT), visual field tests, and electroretinography. A typical case shows a waxy color atrophic optic disc, attenuated retinal arterioles and peripheral intraretinal pigment deposits such as a bone-spicule configuration in the diseased fundus [1]. OCT images of patients with RP show thinning or disappearance of photoreceptor related layers, which proceed toward the center of the retina. The electroretinography of the patients shows a reduction in the rod photoreceptor response and less extent in the cone photoreceptor response. Approximately 100 causative genes for RP have been identified (RetNet; https://sph.uth.edu/retnet/sum-dis.htm#B-diseases, accessed on August 24, 2021). Substitution, deletion, duplication, and/or insertion in genes cause proteins without proper functions, with extra functions, or decrease or disappearance of protein expression. RP is inherited as an autosomal-dominant, autosomal-recessive, or X-linked trait [1], though there are sporadic cases without family history. Many of these genes are involved in the photoreception, phototransduction, and recycling of related visual substances in the photoreceptor and RPE. Because there are many causative genes and there are many cases in which the cause cannot be identified even using the latest genome sequence technology, sufficient elucidation of RP pathophysiology and research and development of therapeutic methods have been delayed.

Mer tyrosine kinase (*MERTK*) is one causative gene of RP [3]. *MERTK* encodes a receptor of the Tyro3/Axl/Mer (TAM) family of tyrosine kinases [4]. TAM receptors are expressed in RPE cells in the eye [5]. MERTK is expressed and localized to the apical tips of RPE cells that penetrate the Photoreceptor Outer Segment (POS) layer and pinch off the distal ends of the OSs [6].

Royal College of Surgeons (RCS) rats have been widely used for several decades as a classic animal model for inherited retinal degeneration with *Mertk* mutation [7-9]. Impairment of phagocytosis of POS by the RPE has been reported in these rats [9]. However, the rat model is not a fully reflected model of human retinal degenerative disorders. Human retinal degeneration carrying a mutation in *MERTK* progresses slowly for several decades; however, this process occurs within months in rats. Moreover, rats lack maculae where cone photoreceptors, which are important for vision and color acceptance, are localized. It is also difficult to obtain raw RPE from patients with RP for research.

The development of induced Pluripotent Stem Cells (iPSCs) has allowed the active research of pathological conditions in monogenic diseases using patient-derived iPSCs [10-12]. RPE cells can be differentiated from iPSCs (iPSC-RPE) efficiently and with high purity [13]. Therefore, in RP, the use of

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iPSC-RPE is optimal for pathological studies in patients with primary lesions in the RPE or mutations in genes that are associated with the RPE [14].

This review highlights pathological studies using iPSC-RPE derived from patients with RP carrying *MERTK* mutations and discusses the possibility of treatment based on previous reports.

Literature Review

MERTK, a causative gene of RP

MERTK was first identified as a causative gene of RP in 2000 [3]. *MERTK* is localized to 2q14.1, consists of 19 exons, and encodes the tyrosine-protein kinase Mer, a 999-amino-acid transmembrane protein. MERTK comprises functional domains: two immunoglobulin-like-C2 (Ig-like C2 type 1 from amino acid (aa) residues 81–186 and Ig-like C2 type 2 aa residues 197-273) and two fibronectin type III (FN-III from aa residues 286 to 381 and 386 to 484) domains in its extracellular portion; and an intracellular tyrosine kinase domain (aa residue 587 to 858) (Figures 1 and 2A) [15]. MERTK belongs to the TAM receptor kinase family. TAM receptors are expressed in dendritic cells, macrophages, and immature natural killer cells of the immune system, sertoli cells of the testis, RPE cells of the eye, and several other types of cells [5]. TAM receptors become heterodimers and bind to their ligands, Growth-Arrest-Specific 6 (GAS6) and protein S. GAS6 and protein S then bind to phosphatidylserine, which is exposed on the extracellular surface of apoptotic cells (Figure 1). TAM receptors remove dead cells resulting from apoptosis. Phosphatidylserine is also exposed on the surface of the POS. In the RPE, MERTK takes up POS into RPE cells by binding the ligands to phosphatidylserine. Thus, this process involves photoreceptor homeostasis of recycling POS and is essential for POS phagocytosis [16,17].

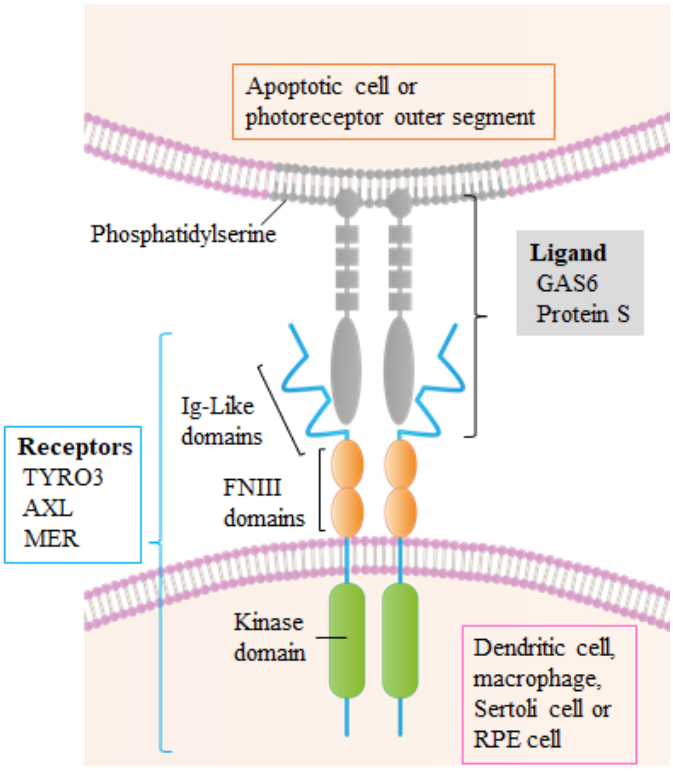

Figure 1. TAM receptors. TYRO3, AXL, and MER (TAM) are receptor tyrosine kinases. TAM receptors have a region with two immunoglobulin (Ig)-like and two fibronectin type III (FNIII) domains in the extracellular region and follow a single-pass transmembrane domain and kinase domains. TAM receptor dimers bind to their ligands, Growth-Arrest-Specific 6 (GAS6), and protein S, through Ig-like domains. GAS6 and protein S bind to phosphatidylserine expressed on apoptotic cells or the photoreceptor outer segment.

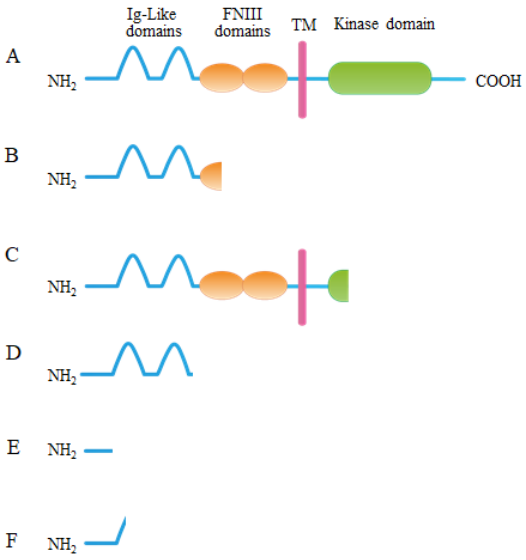

Figure 2. MERTK protein. (A) The wild-type protein has 999 amino acids with immunoglobulin (Ig)-like (residues 81-186 and 197-273), fibronectin type III-like (FNIII) (residues 286-381 and 386-484), transmembrane (TM) (residues 506-526), and kinase (residues 587-858) domains. (B-F) MERTK mutations below result in estimated truncated protein lengths of 334 (B), 650 (C), 77 (E), and 123 (F) amino acids. (B) c.992_993delCA, p.(Ser331Cysfs*5) mutation [19]. (C) c.1951C>T, p.(Arg651*) mutation [20]. (D) Deletion spanning exon 6 to 19, deletion after IgG-like domain (detailed information is not available from the paper [21]). (E) c.225delA, p.(Gly76Glufs*3) mutation [22]. (F) c.370C>T, p.(Gln124*) mutation [22].

Animal models of RP with *MERTK* mutations

The RCS rat is a classic animal model of inherited retinal degeneration [7-9]. In this model, the retina is formed normally; however, rod degeneration begins around 3 weeks after birth and the photoreceptors mostly disappear by 7 weeks of age [7]. The rats are homozygous for a null mutation in *Mertk* [9]. This model has been widely studied and RPE cells have been shown to fail to phagocytose POS and recycle the POS, leading to photoreceptor cell death [9]. As in RCS rats, the phagocytosis of the distal ends of POS was also impaired in *Mertk* knockout mice [18].

In vitro disease model using iPSC-RPE derived from RP patients with *MERTK* mutations

Research using iPSC-RPE from patients with RP and a *MERTK* mutation is expected to develop a model that reflects human pathology. Several groups, including ours, have reported on pathological studies on RP caused by *MERTK* mutations using iPSC-RPE [19-22].

Lukovic, et al. differentiated iPSC-RPE cells from healthy individuals and a patient with RP patient homozygous for the c.992_993delCA, p.(Ser331Cysfs*5) *MERTK* mutation (Figure 2B) [19]. Ramsden, et al. differentiated iPSC-RPE cells from a healthy individual and a patient with RP with compound heterozygous c.61+1G>A/c.1951C>T, p.(Arg651*) *MERTK* variants (Figure 2C) [20]. In each case of iPSC-RPE cells derived from a patient with RP, decreased phagocytosis of fluorescein-5-isothiocyanate (FITC)-POS was observed compared to that in healthy subjects [19,20]. Almedawar, et al. differentiated iPSC-RPE cells of a healthy subject and of a RP patient with a homozygous deletion spanning exon 6 to 19 of *MERTK* (Figure 2D) and evaluated POS ensheathment and phagocytosis in healthy and the diseased iPSC-RPE cells [21]. They also reported that MERTK ligands, such as GAS6 and protein S, stimulate POS ensheathment and phagocytosis and that loss of MERTK function results in the loss of both processes.

We established iPSCs from patients with RP with homozygous c.225delA, p. (Gly76Glufs*3) (Figure 2E) or compound heterozygous c.225delA, p. (Gly76Glufs*3)/c.370C>T, p.(Gln124*) (Figures 2E and 2F)

MERTK mutations and from healthy control subjects [22]. We differentiated the patient and healthy control-derived iPSCs into RPE cells (MTK iPSC-RPE and NOR iPSC-RPE, respectively). Each differentiated iPSC-RPE cell showed a polygonal, cobblestone-like morphology in bright-field micrography. Immunocytochemical staining revealed strong expression of zonula-occludens-1, a tight-junction marker, and polygonal expression of EZRIN, a membrane marker, in both MTK and NOR iPSC-RPE cells. Transmission electron microscopy revealed a monolayer of highly polarized cells with rich apical microvilli and melanosomes in MTK and NOR iPSC-RPE cells. No obvious morphological differences were observed between the MTK and NOR iPSC-RPE cells.

MERTK protein was scarcely detected in MTK iPSC-RPE cells from patients with RP by western blot analysis with an anti-MERTK antibody, although the mRNA expression levels of *MERTK* were comparable between MTK and NOR iPSC-RPE.

To evaluate POS phagocytosis, we added Fluorescein Isothiocyanate (FITC)-labeled POS to iPSC-RPE cells. Using a flow cytometer, we showed reduced internalization of POS in MTK iPSC-RPE cells compared to that in NOR iPSC-RPE cells. We additionally analyzed the amount of a POS-derived protein, rhodopsin, in iPSC-RPE cells fed POS by western blotting. Rhodopsin was detected in NOR iPSC-RPE cells but was hardly detected in MTK iPSC-RPE cells. These findings demonstrated the deteriorated phagocytosis in MTK iPSC-RPE cells compared to that in NOR iPSC-RPE cells.

Thus, these studies have shown that RPE cells differentiated from iPSC established from patients have impaired intracellular uptake of POS.

Possibility of treatment of RP in patients with *MERTK* mutations

Gene transfer: Since *MERTK* mutations were reported to cause retinal degeneration, many gene therapy studies have been carried out using naïve *MERTK* in mouse and rat models. In animal models, gene therapy improved the phagocytic ability of RPE cells and suppressed photoreceptor cell death [23-26].

Ghazi, et al. reported a clinical trial of gene therapy with adeno-associated virus 2-mediated delivery of *MERTK* to patients with RP with *MERTK* mutations [27]. The trial recruited six patients with RP, who were administered a submacular injection of the viral vector and were followed up for 2 years. The therapy was reported to be safe without major side effects and to be effective in some patients.

Translational read-through: Nonsense mutations give rise to Premature Termination Codons (PTCs) in protein-coding regions. These mutations contribute to hereditary diseases because they produce short proteins with little or no functional activity. One treatment strategy for nonsense mutant hereditary diseases is to induce nearly full-length and active protein production by using a read-through compound that skips PTCs. This technology has already been clinically tested for other diseases such as Duchenne muscular dystrophy [28].

Ramsden, et al. reported that the translational readthrough-inducing drugs G418 and PTC124 restored the MERTK expression to detectable full-length protein in iPSC-RPE with *MERTK* mutations that created a stop codon and lack of MERTK expression [20]. PTC124 slightly improved the phagocytic function of diseased iPSC-RPE [20].

Genome editing: The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CAS9 system is a rapid, simple, and effective method for gene editing [29]. This system is a new gene modification technology that can delete, replace, or insert anywhere in the genome sequence by inducing DNA Double-Strand Breaks (DSBs). Artero-Castro, et al. successfully differentiated gene-corrected by CRISPR/CAS9 iPSC-RPE cells derived from a patient with the c.992_993delCA p.(Ser331Cysfs*5) mutation, which causes complete deficiency in MERTK function, and further demonstrated the reestablishment of the expression of full-length MERTK protein and the recovery of lost phagocytic function in the gene-corrected iPSC-RPE cells [30]. Almedawar, et al. reported that the rescue of MERTK expression by CRISPR/Cas9-mediated integration of *MERTK* under the CMV promoter resulted in ensheathment-mediated fragmentation and phagocytosis [21].

Discussion

In this review, we summarized the pathophysiological mechanism elucidated so far of RP with *MERTK* gene abnormality and the current state of therapeutic development research. Studies using animal models and iPSC-RPE have revealed that RPE cells with the gene abnormalities have a reduced ability to take up the POS. Regarding treatments, research and development using new technologies such as gene transfer, translational read-through, and genome editing are underway.

In recent years, with the establishment of iPSC technology and a method for differentiation into RPE, the pathophysiology of monogenic diseases such as RP with *MERTK* gene abnormality and treatment development research have progressed dramatically.

Several groups including us have shown that RPE cells differentiated from iPSC established from patients are deficit in intracellular uptake of POS [19-22]. The patients' truncated MERTK protein without transmembrane and/or tyrosine kinase domains have insufficient protein forms (Figure 2). Loss of function and decreased protein expression of the mutated MERTK protein would result in a loss of function in the PRE cells of patients with RP carrying *MERTK* mutations. While iPSC-RPE is a good *in vitro* model of the disease, there is a limitation that it is difficult to elucidate how impaired POS uptake in RPE causes photoreceptor damage.

We discussed the possibility of RP treatment in patients with *MERTK* mutations. It has already been clarified that the expression of normal MERTK protein using viral vectors improves the ability of RPE cells and suppresses the progression of the disease in animal models [23-26]. The clinical trial of gene therapy with adeno-associated virus 2-mediated delivery of *MERTK* to patients with RP carrying *MERTK* mutations reported the treatment to be safe without major side effects and to be effective in some patients [27]. However, it may take some time before gene therapy is employed for practical use.

Research using translational read-through technology is also being conducted using iPSC-RPE. The mechanism of action of translational readthrough-inducing drugs has not been completely elucidated. Drug therapy targeting PTC nonsense mutations, regardless of the causative gene, is expected. Clinical test has already begun for other diseases. Though the indication for this type of drug therapy is limited to cases with mutations that create a stop codon, it is expected that this technology will also be applied to RP depending on the type of gene mutation.

The CRISPR/CAS9 system is another attractive method for therapies and disease modeling. Studies using iPSC-RPE have revealed that using the system it has succeeded in expressing a functional MERTK protein. Promotion of the research and future clinical application is desired.

No effective treatment for RP has yet been established. iPSC-RPE cells derived from patients with RP have been established and used as a useful and powerful research tool to elucidate the pathological mechanism and develop treatments, including gene therapy. Although further research is needed, targeted genome manipulation, such as translational read-through and genome editing therapies may be a useful future treatment for patients with RP.

Conclusion

We reviewed and focused on the function of phagocytosis of iPSC-RPE cells derived from patients with RP carrying *MERTK* mutations and discussed the possibility of treatment development. In the diseased RPE, intracellular uptake of POS was impaired. We further and discussed the

possibility of treatment development including gene therapy and gene editing. iPSC-RPE derived from patients is a useful research tool not only for elucidating the pathological mechanism but also for developing treatments, though it may take some time before treatments are put into practical use.

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Conflict of Interest

The authors have no conflict of interest to declare.

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