Osteopetrosis associated with PLEKHM1 and SNX10 genes, both involved in osteoclast vesicular trafficking

Osteopetrosis associated with *PLEKHM1* and *SNX10* genes, both involved in osteoclast vesicular trafficking

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Highlights

- Autosomal recessive osteopetrosis is genetically heterogeneous
- Different pathogenic mechanisms can cause autosomal recessive osteopetrosis
- Pathogenic variants in *PLEKHM1* and *SNX10* can underly autosomal recessive osteopetrosis

Abstract

The clinical and radiological variability seen in different forms of osteopetrosis, all due to impaired osteoclastic bone resorption, is reflected in the extended set of genes that has been shown to be involved. Both differentiation of osteoclasts from hematopoietic stem cells as well as disturbance at the level of the functioning of the osteoclasts can be the underlying pathogenic mechanism. Pathogenic variants in *PLEKHM1* and *SNX10* can be classified among the latter as they impair vesicular transport within the osteoclast and therefore result in the absence of a ruffled border. Radiologically, some of the typical hallmarks of osteopetrosis can be seen and most cases present with a relatively mild form segregating in an autosomal recessive mode of inheritance.

Introduction

Throughout life, bone is permanently renewed by the process of bone remodeling, involving bone resorption by osteoclasts and subsequent bone formation by osteoblasts. The regulation and balancing of both processes, partially performed by osteocytes, are essential to avoid clinical complications. The most prevalent example of such a clinical entity is osteoporosis in which a decline in bone mass results in increased bone fragility. Towards the other end of the spectrum, there are a number of conditions, often monogenic diseases, that are characterized by an increased bone mass and therefore called the sclerosing bone dysplasias [1]. This group of conditions is very heterogeneous at the clinical, radiological and molecular genetic level. In many of these conditions, secondary effects including anemia, hepatosplenomegaly, cranial nerve compressions resulting in facial nerve palsy, hearing and vision problems, can occur. Despite the fact that there is an increase in bone mass, in some conditions patients have an increased fracture risk because the microscopic structure of the bone tissue is disturbed, resulting in a decreased strength of the bones. This is definitely the case for the osteopetroses [2]. This group of conditions share their pathogenic mechanism as in all of them the disease is caused by impaired osteoclastic bone resorption. This results in the presence of remnants of

unresorbed cartilage in the bone tissue which is the histopathological hallmark of these conditions resulting in decreased resistance to fractures.

Over the last two decades, a lot of insights have been generated on the pathogenic mechanisms underlying the osteopetroses with the identification of a set of genes involved in the different subtypes of this disease [2]. Some of the genes and encoded proteins turned out to be essential for the differentiation of hematopoietic stem cells towards multinucleated osteoclasts. This is the case for the tumor necrosis factor receptor superfamily, member 11A (*TNFRSF11A*) and *TNFSF11* genes encoding respectively the receptor activator of NF-κB signaling (RANK) and its ligand RANKL. Both proteins play a role in NF-κB signaling which is an essential pathway for osteoclast differentiation. Therefore, loss of function of these proteins results in an osteoclast-poor form of osteopetrosis [3].

For another set of genes identified, the encoded proteins turned out to be involved in the process of acidification of the extracellular compartment between the osteoclast and the bone tissue. An acidic environment at the site of bone resorption is essential to have this process taken place. Carbonic anhydrase II generates protons that are translocated over the membrane by a proton pump of which ATP6i (encoded by the *TCIRG1* gene) is an essential subunit [4]. To keep the process going, the generated potential is compensated by transfer of chloride anion by the ClCN7 proton pump and its β-subunit OSTM1 [5-7]. Pathogenic loss of function variants in all the genes mentioned underly different forms of osteopetrosis.

In this review we focus exclusively on two other genes, *PLEKHM1* and *SNX10,* that can upon genetic variation result in other autosomal recessive forms of osteopetrosis (ARO). As will be shown, there is clinical and radiological overlap with other forms but the pathogenic mechanism is caused by disturbance of intracellular vesicular transport.

Plekhm1

Genetics

By a positional cloning effort in the *incisors absent (ia)* rat, a spontaneous osteopetrotic rat mutant first described in 1941 [8], we were able to identify a homozygous deletion of 1 cytosine in exon 4 of the Pleckstrin homology domain-containing protein, family M, member 1 (*PLEKHM1*) gene (OMIM 611466)[9]. This variant results in a frameshift and an early stop codon, and therefore a loss of function effect is assumed. Within the same study, a homozygous splice site variant at the splice donor site of intron 3 was found in a patient diagnosed with an autosomal recessive form of osteopetrosis (Figure 1)[9].

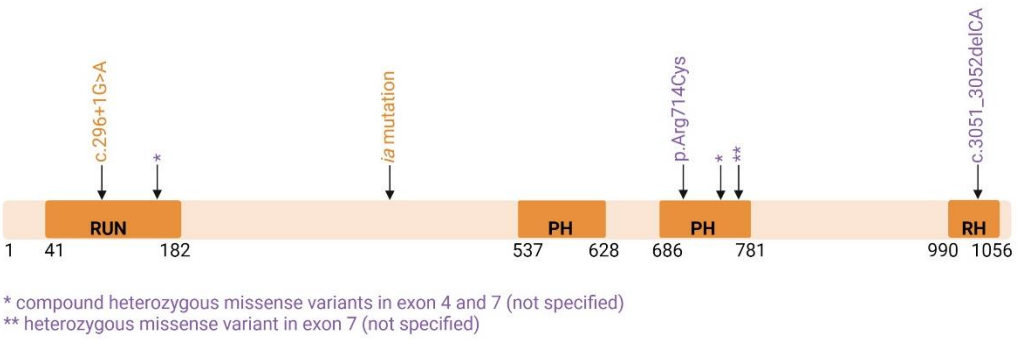

FIGURE 1. Overview of PLEKHM1 and the reported pathogenic variants in PLEKHM1-related ARO. The functional domain of PLEKHM1 includes a RUN domain, two Pleckstrin Homology (PH) domains

and a Rubicon Homology (RH) domain. The variants are heterozygous (purple) or homozygous (orange).

Del Fattore *et al.* identified a heterozygous variant at position 2140 of the *PLEKHM1* gene inducing a p.Arg714Cys aminoacid substitution (Figure 1)[10]. The patient was diagnosed with osteopetrosis at the skull and the remainder of the skeleton showed a complex bone phenotype of generalized osteopenia with some areas of focal osteosclerosis.

In 2016, Bo *et al.* reported a patient with a history of fractures, early tooth loss, anemia and hepatosplenomegaly, as well as increased bone mineral density. Based on this, he was diagnosed with osteopetrosis [11]. They identified a *de novo* heterozygous *PLEKHM1* variant, a CA deletion in exon 11 (c3051_3052delCA), resulting in a frameshift disturbing the Rubicon Homology (RH) domain (Figure 1).

In a case report, Moore *et al.* reported on a 19-year old male patient with a history of multiple fractures and radiological evidence for osteopetrosis. The patient turned out to be compound heterozygous for 2 variants in exon 4 and 7 (Figure 1). However the precise nature of these variants was not revealed [12]. Similarly, Almarzooqi *et al.* reported a newborn with a heterozygous variant in exon 7 of the *PLEKHM1* gene [13]. The diagnosis was made based on the presence of hepatosplenomegaly and histologic evaluation showing abnormally thickened bony trabeculae. However, the same (not specified) variant was also found in the healthy mother.

Clinical and radiological aspects

As only very few variants are currently identified, it is difficult to get a precise view on the clinical and radiological aspects of this form of osteopetrosis. In some cases the disease segregates in an autosomal recessive mode of inheritance [9, 12], and therefore a loss of function effect is assumed. The patients involved show an intermediate form of osteopetrosis including an Erlenmeyer flask deformity and chondrolysis of the hip in one case [9], while the other showed a rugger jersey spine appearance and suffered from multiple fractures [12]. No biochemical abnormalities were measured.

In some other cases, only one heterozygous variant was identified. Bo *et al.* identified a *de novo* frameshift variant resulting in impaired functioning of the encoded PLEKHM1 [11]. A dominant negative effect of this mutation cannot be excluded. The patient showed a typical radiological picture of generalized sclerosis with sandwich appearances of the vertebrae. Multiple fractures, as well as pancytopenia and hepatosplenomegaly were present. The missense variant p.Arg714Cys results in an generalized osteopenia, although in some areas osteosclerosis is seen [10]. As the urine CTX levels were normal and the *in vitro* analysis of the osteoclasts did not show any abnormalities, it is questionable whether a diagnosis of osteopetrosis is appropriate for this patient. Finally, a heterozygous variant was reported in a newborn with hepatosplenomegaly and pancytopenia and increased thickness of the trabeculae [13]. However, as the variant was inherited from the healthy mother and as no radiological evidence was available at the very young age of the patient to confirm the diagnosis of osteopetrosis, follow up of this newborn is needed to decide whether the diagnosis of PLEKHM1-induced osteopetrosis can be confirmed.

Osteoclast phenotype and pathogenesis

Evidence from studies on the human pathogenic variants, as well as on the *ia* rat model, support the conclusion that PLEKHM1 is not involved in osteoclast differentiation, but rather in the functioning of mature osteoclasts. Cultured osteoclasts do not show evidence of bone resorption explained by the absence of a ruffled border which is essential for bone resorption.

Colocalization studies indicated the presence of PLEKHM1 in late endosomes/lysosomes based on colocalization with RAB7 [9](Figure 2). This binding with RAB7 is generated most likely via the RUN domain for which interaction with the Ras-like small GTPases has been reported before [14, 15]. The Rab family are known to be important regulators of vesicular transport with an essential role for RAB7 in osteoclastic bone resorption [16].

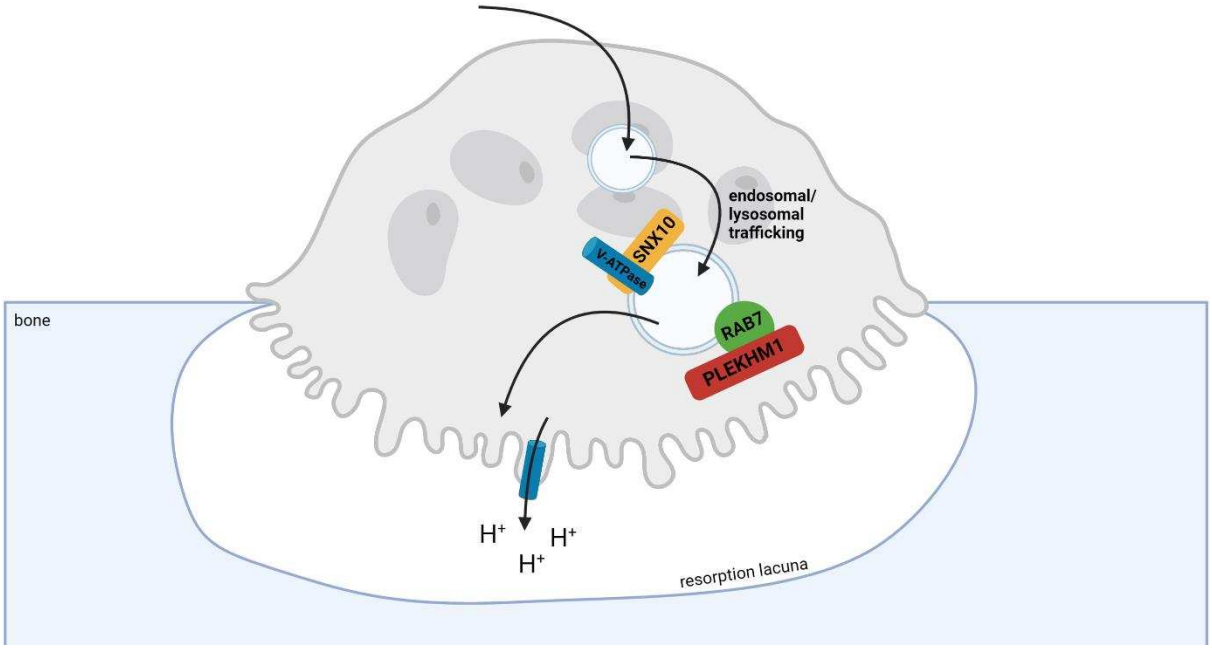

FIGURE 2. The role of PLEKHM1 and SNX10 in osteoclast vesicular trafficking. Normal osteoclast functioning is dependent on endosomal/lysosomal vesicular trafficking. Both PLEKHM1 and SNX10 are major players in this process. PLEKHM1 binds with RAB7, an essential regulator of osteoclast vesicular transport. Regarding SNX10, an interaction with vacuolar-type H^+-ATPase (V-ATPase), has been demonstrated. Vesicular transport of this V-ATPase to the ruffled border is crucial for the formation of this ruffled border and acidification of the resorption lacuna.

Further studies identified additional interaction partners of PLEKHM1, several of them corroborating its role in vesicular transport, ruffled border formation and therefore bone resorption. Witwicka *et al*. illustrated the interaction with TRAFD1 (FLN29)[17]. Knockdown of the latter inhibited acidification of the resorption lacuna and therefore bone resorption. In 2017, Marwaha *et al.* reported the binding between the RUN domain of PLEKHM1 and Arl8b, another small GTPase [18]. In addition, evidence was generated that PLEKHM1 plays a role in connecting lysosome to microtubules and therefore the positioning and secretion of the lysosomes through RAB7. Interaction with DEF8 is essential for its binding to RAB7, while complexing with FAM98A and NDEL1 makes the connection with microtubules possible [19].

Disease models

The osteoclasts from the *ia* rat exhibit ultrastructurally extended clear zones where attachment to the bone takes place, at the expense of ordinary ruffled borders, indicating an intrinsic defect in ruffled border formation. Furthermore, a secretory dysfunction in the mutant is strongly suggested by the absence of detectable extracellular tartrate-resistant acid phosphatase (TRAP) while there is an accumulation of the enzyme in abundant small cytoplasmic vesicles [20].

The use of germline and conditional *Plekhm1* knockout models resulted in an increased cancellous bone mass due to decreased bone resorption [19]. There was no indication for an effect on osteoclast differentiation. Osteoblast numbers and bone formation rate were reduced but this is likely secondary to coupling with reduced bone resorption. *In vitro* studies showed that PLEKHM1 regulates the peripheral distribution of lysosomes and their secretion.

SNX10

Genetics

Over the past decade, the *SNX10* gene (OMIM 614780), encoding sortin nexin 10, has become an interesting player in the etiology of autosomal recessive osteopetrosis (ARO). It is currently estimated that 4-5% of ARO cases are caused by pathogenic variants in *SNX10* [2, 21]. In 2012, a first missense variant, i.e. c.152G>A; p.Arg51Gln, was identified in members of three consanguineous Palestinian families by Aker *et al*. [22]. Since then, 13 additional bi-allelic loss-of-function variants, including nonsense, missense and splicing variants, have been reported within several studies (Figure 3)[21, 23-29]. Among these variants is a frameshift variant (c.212+1G>T) that was identified in patients suffering from Västerbottenian osteopetrosis, which refers to a cluster of cases in Västerbotten County (Sweden) with an increased disease incidence due to a founder effect [21, 24]. Current data show that ARO caused by *SNX10* variants is phenotypically heterogeneous [30, 31]. This may be due to incomplete penetrance or variable genetic expression, as no genotype/phenotype correlations could be established [21, 32].

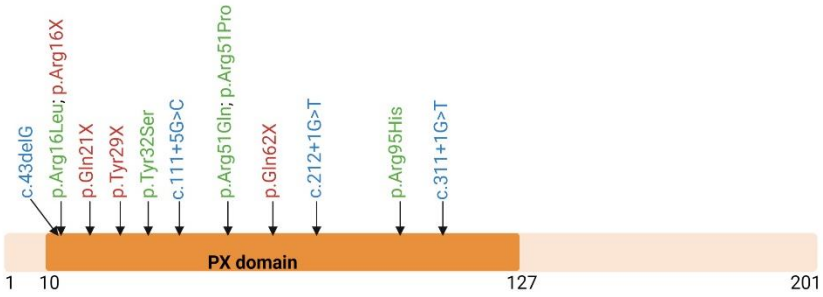

FIGURE 3. Overview of SNX10 and the reported pathogenic variants in *SNX10*-related ARO. The functional domain of SNX10 is the Phox homology (PX) domain, in which all pathogenic variants in *SNX10*-dependent ARO patients are located. These variants are homozygous missense (green), nonsense (red) or splicing (blue) variants that result in a loss of protein function. In addition, a deletion of approximately 70 kb (7p15.2, hg18 chr7:g.(26249558_26251671)-(26321193_26322492)del) upstream of *SNX10* was identified (not shown).

Clinical and radiological aspects

The large majority of patients exhibit osteoclast-rich osteopetrosis in combination with secondary anemia and visual impairment [21, 22, 27, 31, 33, 34]. In addition, hepatosplenomegaly, hearing loss, mild growth retardation and dental problems are frequently observed [31, 33]. According to a previous study based on radiological examination, *SNX10*-related ARO generally manifests a relatively milder disease course, at least compared to the other causal genes examined, such as *TCIRG1* [35].

In all patients, the disease manifested in early infancy with a disease onset at less than 1 year of age, and the life expectancy varies from 0 to 22 years [33, 36]. Since none of the patients has immunological problems and/or primary neurodegeneration, a hematopoietic stem cell transplantation (HSCT) can serve as an effective therapy in severe cases [21, 27, 33, 37]. For this purpose, allogeneic hematopoietic

stem cells are transplanted and eventually develop into normally functioning osteoclasts, so that the phenotype can be (almost) completely rescued, provided that treatment is started as soon as possible [22, 32, 33]. Nonetheless, there are many disadvantages associated with HSCT, making further research into novel anti-resorptive therapies of major relevance [37]. For a long time, SNX10 was considered an interesting candidate target, as its main site of expression is the osteoclast and as all symptoms observed in patients with *SNX10* variants can be linked to the bone phenotype [21, 33, 36]. However, there is emerging evidence that SNX10 plays a role outside the skeletal system, i.e. in cancer, metabolic diseases and chaperone-mediated autophagy [30, 38-40].

Osteoclast phenotype and pathogenesis

SNX10 belongs to the sortin nexin family of proteins, characterized by the presence of a conserved phospholipid-binding Phox homology (PX) domain and well-known for its function in vesicular trafficking, protein sorting and endosomal homeostasis [41, 42]. The PX domain of SNX10 predominantly binds to phosphatidylinositol 3-phosphate (PI3P), thereby targeting SNX10 to the membrane of early endosomes [43]. This finding is in agreement with later studies, which contributed to elucidate the involvement of SNX10 in endosomal pathways by identifying specific binding partners of SNX10 [44-47].

Considering all data, it is not surprising that pathogenic variants in *SNX10* result in the development of an osteopetrosis phenotype. Vesicular trafficking via the endosomal/lysosomal pathway is required for the normal function of the osteoclast, e.g. for the formation of the ruffled border or the so-called resorptive organelle of the osteoclast. The ruffled border is adjacent to the bone surface, and is formed and maintained by trafficking of endocytic vesicles originating from the basolateral membrane of the osteoclast [48-51]. Moreover, the ruffled border is enriched in vacuolar-type H^+-ATPase (V-ATPase), which drives the acidification of the underlying resorption lacuna, in order to break down the mineral bone matrix [49-51]. Previously, Chen *et al.* demonstrated that SNX10 directly interacts with the V1D subunit of V-ATPase [52]. In combination with the known role of sortin nexins in protein sorting, these results indicate that SNX10 is an essential player in the delivery of V-ATPase to the ruffled border (Figure 2). Pathogenic variants in *SNX10* will therefore inevitably lead to failure of ruffled border formation, lack of acidification of the subosteoclastic region and dysfunctional bone resorption.

To date, conflicting findings have been reported regarding the histopathological situation in the *SNX10* mutated subgroup of ARO patients. In their original work, Aker *et al.* described that a pathogenic missense variant resulted in the presence of fewer and smaller osteoclasts, with considerably reduced resorptive capacity [22]. In addition, the authors noted large endosomal vacuoles, very similar to autophagolysosomes, indicating that the endosomal pathways were severely disrupted. Interestingly, the involvement of SNX10 in endosome homeostasis is supported by the previous finding that overexpression of exogenous SNX10 results in the formation of giant vacuoles in mammalian cells [43].

In contrast, in a more recent paper, the patient's osteoclasts were larger, pale after TRAP staining and there was no evidence of vacuolization [24]. Although the authors attributed this observation to increased spreading rather than increased fusion, it was recently shown in $Snx10^{Arg51Gln/Arg51Gln}$ mice that an unknown SNX10-dependent mechanism can result in continuous cell-cell fusion and subsequent formation of extremely large osteoclasts [53].

However, it is worth noting that all studies have consistently shown that there is no evidence of bone resorption, demonstrated for example by the absence of both the resorption pits and the release of carboxy-terminal collagen crosslinks (CTX) in osteoclasts from patients with *SNX10* variants [22, 24]. This lack of resorption is a direct consequence of a defective formation of the ruffled border, in which

the endosomal/lysosomal trafficking pathways are of major importance [24, 48]. Taken all together, it is clear that genetic variation in *SNX10* can lead to the development of osteoclast-rich osteopetrosis.

Disease models

The growing interest in unraveling the underlying disease mechanisms has led to the creation of multiple SNX10-associated mouse models. The first model was generated by Ye *et al*. and was based on a global *Snx10*-defiency, resulting in an osteopetrosis phenotype combined with rickets [54]. The latter is a clinical feature not yet reported in human patients, and is presumably a consequence of decreased bone mineralization following impaired gastric acidification and low calcium absorption. Furthermore, the mice died shortly after birth and showed no ruffled border or acidification of resorption lacunae. Interestingly, an osteoclast-specific *Snx10* knockout model showed a severe osteopetrosis phenotype, with no rickets involved [54]. A similar phenotype was observed in an *Snx10* knock-in mouse model ($Snx10^{Arg51Gln/Arg51Gln}$), thus indicating the presence of a severe, early-onset osteopetrosis phenotype [55]. Again, the mice failed to form a ruffled border or to secrete protons.

Whilst these mouse models have provided new insights into the disease course of *SNX10*-dependent ARO, other models are also being developed in order to increase research possibilities, such as patient-derived induced pluripotent stem cells (iPSC). In this regard, the generation of an iPSC cell line from a patient with Västerbottenian osteopetrosis, thus harboring the pathogenic c.212+1G>T variant, is of particular relevance [56].

Discussion

Unraveling the pathogenic mechanisms underlying the heterogeneous group of osteopetroses resulted in evidence for causality of variants in some genes involved in osteoclast differentiation. In addition, impaired acidification of the extracellular compartment was identified as the pathogenic mechanism in other forms of osteopetrosis. Finally, also pathogenic variants in *PLEKHM1* and *SNX10* were identified. This is not to be considered remarkable as both play an important role in vesicular transport in the osteoclast, which is essential for the formation and maintenance of the ruffled border where bone resorption takes place.

For the *PLEKHM1* gene only few variants are described and therefore a genotype-phenotype correlation is difficult to be made. For some of the variants further studies are needed to confirm their causality. It has to be mentioned that upon mutation screening of the *PLEKHM1* gene, one should take into account the presence of a pseudogene located next to the functional gene as apparently heterozygous variants can be explained by sequence variations in the pseudogene. Pathogenic variants in *SNX10* result in a variable picture, but in most cases early signs make an early diagnosis/prognosis possible.

In general, it looks like variants in any of both genes can result in autosomal recessive forms of osteopetrosis that can be diagnosed in early infancy. In comparison with other forms of ARO, the patients show relatively mild features. There is currently no specific treatment available for these forms of osteopetrosis. However, for severe cases, HSCT can be considered as the molecular defects are intrinsic to osteoclast cells themselves.

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