in vivo *24*: 309-314 (2010)

Milroy's Primary Congenital Lymphedema in a Male Infant and Review of the Literature

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Abstract. *Background: Milroy's primary congenital lymphedema is a non-syndromic primary lymphedema caused mainly by autosomal dominant mutations in the FLT4 (VEGFR3) gene. Here, we report on a 6-month-old boy with congenital non-syndromic bilateral lymphedema at both feet and tibias, who underwent molecular investigation, consisted of PCR amplification and DHPLC analysis of exons 17-26 of the FLT4 gene. The clinical diagnosis of Milroy disease was confirmed by molecular analysis showing the c.3109G>C mutation in the FLT4 gene, inherited from the asymptomatic father. This is a known missense mutation, which substitutes an aspartic acid into a histidine on amino acid position 1037 of the resulting protein (p.D1037H), described in two other families with Milroy disease. A thorough genetic molecular investigation and clinical evaluation contributes to the provision of proper genetic counseling for parents of an affected child with Milroy disease. The herein described case, which is the third reported so far with c.3109G>C mutation, adds data on genotypic-phenotypic correlation of Milroy disease. The relative literature regarding the pathophysiology, molecular basis, clinical spectrum and treatment of Milroy disease is reviewed.*

Milroy's primary congenital lymphedema (also known as Nonne-Milroy lymphedema, hereditary lymphedema I, and Milroy disease, OMIM 153100) is a developmental disorder of the lymphatic system which leads to disabling and disfiguring swelling of the extremities (1). The swelling varies in degree and distribution. Limb swelling of all extremities may occur, while bilateral but asymmetric lower-limb lymphedema is most typical. Other features include prominent veins, cellulitis, hydrocele, papillomatosis, and typical upslanting 'ski-jump' toenails. Lymphedema might be present at birth or developing soon after, and usually the degree of edema progresses. There is wide inter- and intrafamilial clinical variability, including cases with prenatal manifestation evolving to hydrops fetalis, as well as mild cases with first presentation at the age of 55 years in asymptomatic individuals (1-5). Milroy's disease was first described by William Forsyth Milroy in 1892, and since then more than 300 patients have been reported (6, 7). It is a relatively rare condition with an estimated frequency of approximately 1:6000 births, and a male/female ratio of 1/2.3 (3, 8).

Milroy's disease shows an autosomal dominant pattern of inheritance with reduced penetrance of 80-90% (9). A first type of Milroy's disease has been mapped to the telomeric part of chromosome 5q (5q34-q35) in several families (10), whereas a second locus was mapped to chromosome 6q (6q16.2-q22.1 in a single inbred Pakistani family (5). Ferrell *et al.* (1) were the first to identify a mutation in Milroy's disease in the *FLT4* gene (also known as *VEGFR3*) located in 5q34-q35. Later, more *FTL4* mutations were identified (8-20). In a larger study of patients with Milroy's disease, Connell *et al.* (18) found an *FTL4* mutation in 75% of typical Milroy patients with a positive family history, and 68% of sporadic patients (18). The likelihood of detecting *FLT4* mutations in patients who have a phenotype which is not typical of Milroy's disease (lower limb lymphedema, unilateral or bilateral, but symptoms not noted at birth or presence of other features not previously associated with Milroy's disease) is very small (<5%). Most *FLT4* mutations are autosomal dominant, but a single homozygous hypomorphic mutation has been found in an autosomal recessive form of non-syndromic primary congenital lymphoedema (21).

The *FLT4* gene encodes a receptor tyrosine kinase, Fms-like tyrosine kinase 4 (or vascular endothelial growth factor receptor 3) specific for lymphatic vessels. The gene is

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Key Words: Congenital lymphedema, *FLT4* gene, Milroy disease.

composed of 31 exons transcribed as two alternatively-spliced transcripts encoding proteins with 7 immunoglobulin-like repeat domains and 2 tyrosine kinase domains (15). The gene is a member of the platelet-derived growth factor receptor subfamily of class III receptor tyrosine kinases (22). *VEGFR* genes participate in several processes essential in angiogenesis and lymphangiogenesis, including endothelial cell migration, proliferation and survival (22). FLT4/VEGFR3 appears to be the most critical of these receptors in lymphatic development (22).

We report on a 6-month-old boy with Milroy's disease due to a known heterozygous c.3109G>C (p.D1037H) missense mutation in the *FLT4/VEGFR3* gene, and review the literature on this disorder.

Patient and Methods

At the age of 6 months, the proband was referred to the Department of Medical Genetics because of persistent edema of both feet. The patient is the first child of two unrelated and phenotypically normal parents of Greek origin. There was a previous miscarriage at 26 weeks gestation. At autopsy, that fetus had several morphological abnormalities, including hypertelorism, low set ears, and bulbous nose. No major congenital anomalies were reported in the autopsy results, apart from severe stenosis of the small intestine and partial meconium obstruction. The limbs were macroscopically normal. Death was attributed to intrauterine anoxia as was concluded from the appearance of lung symphoresis and aspiration. Karyotype analysis and molecular analysis of the complete open reading frame of the *CFTR* gene were normal. No formal diagnosis could be made.

In the second gestation, fetal ultrasound was normal but chromosomal analysis after amniocentesis was performed because of parental anxiety due to the previous miscarriage and showed a normal male karyotype. The proband was born by vaginal delivery after an uneventful 39-weeks' gestation with a birth weight of 4050 g, length of 51 cm and head circumference of 32 cm (all within 90th-97th percentile). At birth, he presented with dorsal edema of both feet with upslanting toenails. A heart murmur was present, but ultrasonographic investigation of the heart only showed a minor atrial septal defect due to fenestration of the septum secundum. Apart from these, the neonate did not have any other congenital malformations, and the postnatal period was uneventful.

Clinical evaluation at the age of 6 months showed normal growth and psychomotor development. Discrete bilateral swelling of both lower limbs up to the knees with soft browny edema was noticed, without prominent veins, papillomatosis or cellulitis (Figure 1). Radiological examination of both lower limbs excluded any skeletal deformity. Ultrasonography showed normal morphology and patency of the major arteries, veins and their branches bilaterally with only a few veins dilated at the dorsal site of the ankle joint (Figure 1). The findings suggested edema of the subcutaneous tissue but without any other vascular dysplasia. The patient was free of any other pathological findings from other systems, thus his lymphedema was clinically similar to that of Milroy's disease.

The molecular investigation consisted of PCR amplification and DHPLC analysis of exons 17-26 of the *FLT4* gene.

Results

Molecular analysis of the *FTL4* gene showed a heterozygous c.3109G>C mutation in exon 23. This mutation substitutes an aspartic acid into a histidine on amino acid position 1037 of the resulting protein (p.D1037H). The c.3109G>C mutation is a known *FTL4* mutation described in other patients with Milroy's disease, as previously described by Connell *et al*. 2009 (18) (Table I). Subsequent sequencing of exon 23 of the *FTL4* gene in the parents, as requested for genetic counseling purposes, showed that the proband´s father carried the same mutation despite his normal phenotype. Furthermore, there was no history of congenital limb swelling. The paternal grandparents were reported to be nonaffected, but unfortunately they were not available for clinical or molecular studies.

Discussion

Lymphatic vessels play a central role in maintaining interstitial fluid balance. The development of the human lymphatic vascular system begins in the sixth to seventh week of embryonic life, nearly one month after the development of the first blood vessels. Angiogenesis and lymphangiogenesis are tightly regulated by growth factors, intercellular and cell-ECM signaling mechanisms. Endothelial cell fate, on the other hand, is determined by a large number of different signals, among which some are simultaneously transduced by numerous ligand-tyrosine kinase receptor systems as the vascular endothelial growth factor (VEGF), angiopoietin, PDGF and TGF-β families (23).

Lymphedema is characterized by a chronic disabling swelling of the extremities caused by an increase in the interstitial protein rich fluid, which subsequently results in insufficient lymphatic transport and drainage (15, 20, 24). The majority of patients with lymphedema can be diagnosed through thorough history-taking, physical examination and ultrasound. Lymphatic visualization may be indicated to confirm the diagnosis. Isotopic lymphoscintigraphy is generally considered as the gold standard for the diagnosis of lymphedema, as the procedure is minimally invasive, easy to perform and harmless to the lymphatic endothelium (24, 25). Fluoresence microlymphography is a practically atraumatic technique used to visualize the superficial skin network of initial lymphatics through the intact skin of man and can also measure the microlymphatic pressure and velocity (26). Direct lymphography is essential for providing more anatomical details (24). CT imaging has been shown to be highly sensitive (97%) and specific (100%). Although more costly, MRI offers greater detail of lymphatic architecture, and confers no radiation exposure.

There are two major categories of lymphedema: primary (idiopathic) and secondary (acquired), the latter with a

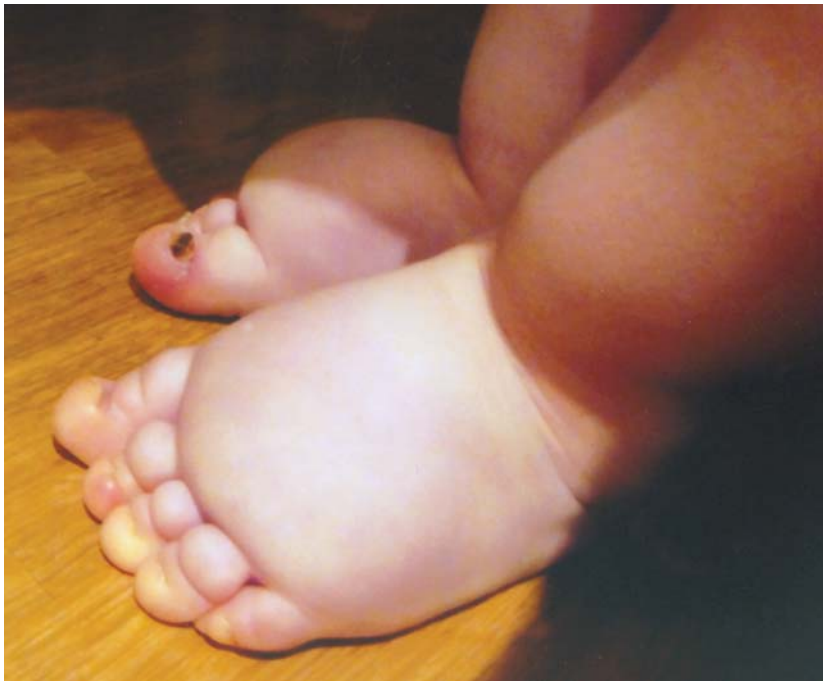

Figure 1. Bilateral primary lymphedema of the lower limbs along with upslanting toenails.

known pathogenic alteration (20). In primary lymphedema, which can be either isolated or in association with other clinical problems as well as part of a defined syndrome, lymphatic vessels can be either hypoplastic or hyperplastic, but are nonfunctional. In all types of lymphedema, there is abnormal accumulation of interstitial protein-rich fluid caused by a congenital malformation (primary lymphedema) or as a result of lymphatic obstruction or disruption (secondary lymphedema) of the lymphatic vessels (22-24). As our patient had congenital lymphedema, primary lymphedema was diagnosed.

There are several genetic causes of primary lymphedema. Turner's syndrome, which in newborn girls may present with bilateral puffiness of the hands and feet and redundant nuchal folds, was excluded in our patient as his external genitalia were male and his karyotype was 46, XY. In Noonan syndrome lymphedema may present prenatally and/ or postnatally, which can be localized or widespread, and dorsal limb lymphedema is most common (24). However, the lack of any congenital heart defects and facial anomalies ruled out Noonan syndrome in our patient (22, 24). In lymphedema-distichiasis syndrome (OMIM 153400), an autosomal dominant disorder caused by *FOXC2* mutations, the congenital lymphedema is associated with a characteristic double row of lashes (4, 22, 24), which was not present in our patient. Lymphedema and ptosis syndrome (OMIM 153000) and yellow nail syndrome (OMIM 153300) (4) were excluded in our patient as he had no ptosis, nor nail discoloration. Hypotrichosis-lymphedema-telangiectasia syndrome (OMIM 607823) caused by a *SOX18* mutation (4) is unlikely in our proband as his hair is not sparse and there are no cutaneous telangiectasias of palms and soles. Our patient also did not meet any of the cardinal features (hypohidrosis and hypotrichosis) of the rare syndrome osteoporosis-lymphedema-anhydrotic ectodermal dysplasia with immunoinsufficiency (OMIM 300301) or Aagenaes syndrome associated with lymphedema and neonatal cholestasis (4, 6, 22, 24). As our patient had congenital primary lymphedema without any of the characteristic symptoms of the syndromes described above, Milroy's disease was diagnosed. This was confirmed by molecular analysis showing a c.3109G>C mutation in the *FLT4/VEGFR3* gene, the disease gene implicated in Milroy's disease.

Milroy's disease is a relatively rare form of primary lymphedema usually caused by a mutation in the *FLT4/VEGFR3* gene. In affected individuals, the superficial lymphatic vessels of the affected edematous areas are thought to be hypoplastic or aplastic (3, 22, 23, 26). However, in a recent study of 71 cases of Milroy's disease with identified *FLT4/VEGFR3* mutation, skin biopsy from the swollen feet of the patients revealed the presence of abundant skin lymphatic vessels, which however were non-functional on fluorescence microlymphangiography (7). A high prevalence of superficial venous reflux was detected,

Table I. *Overview of FLT4 mutations reported in the literature.*

Mutation	Domain	Familial/ sporadic	Phenotype	Reference (no.)
c.2531G>C (p.R844P)	TKI	Familial	Classical (1 case diagnosed *in utero*)	Connell *et al*., 2009 (18)
c.2554G>A (p.G852S)	TKI	Familial	Classical	Connell *et al*., 2009 (18)
c.2554G>C (p.G852R)	TKJ	Familial	Classical	Connell *et al*., 2009 (18)
c.2560G>A (p.G854S)	TKI	Familial	Classical	Evans *et al*., 2003 (12)
c.2561G>C (p.G854R)	TKI	Familial	Classical	Connell *et al*., 2009 (18)
c.2564G>A (p.A855T)	TKI	Sporadic	Autosomal recessive primary congenital lymphoedema	Ghalamkarpour *et al*., 2009a (20)
c.2569G>A (p.G857R)	TKI	Familial	Classical	Karkkainen *et al*., 2000, Mizuno *et al*., 2005 (11,13)
c.2632G>A (p.V878M)	TKI	Familial	Classical (1 case diagnosed *in utero*)	Ghalamkarpour *et al*., 2006 (8)
c.2677C>G (p.L893V)	TKI	Sporadic	Classical (1 case diagnosed *in utero*)	Ghalamkarpour *et al*., 2009b (21)
c.2743G>C (p.A915P)	TKI	Familial	Classical	Evans *et al*., 2003 (12)
c.2748C>G (p.C916W)	TKI	Familial	Classical	Evans *et al*., 2003 (12)
c.2797G>C (p.G933R)	TKI	Familial	Classical (1 case diagnosed *in utero*)	Evans *et al*., 2003, Connell *et al*., 2009 (12,18)
c.2828G>C (p.R943P)	TKI	Familial	Classical	Connell *et al*., 2009 (18)
c.3059A>T (p.Q1020L)	TKII	Familial	Classical	Butler *et al*., 2007 (15)
(p.G1024Q)	TKII	Familial	Classical	Connell et al, 2009 (18)
(p.G1024R)	TKII	Familial	Classical	Connell *et al*., 2009 (18)
c.3104A>G (p.H1035R)	TKII	Familial	Classical	Irrthum *et al*., 2000 (10)
c.3105C>G (p.H1035Q)	TKII	Sporadic	Classical	Ghalamkarpour *et al*., 2006 (8)
c.3109G>T (p.D1037Y)	TKII	Familial	Classical	Connell *et al*., 2009 (18)
c.3109G>C (p.D1037H)	TKII	Familial	Classical	Connell *et al*., 2009 (18)
c.3121C>T (p.R1041W)	TKII	Classical		Evans *et al*., 2003, Carver *et al*. 2007, Connell *et al*., 2009 (12,7,18)
c.3122G>A (p.R1041Q)	TKII		Classical	Evans *et al*., 2003, Carver *et al*. 2007, Connell *et al*., 2009 (12,7,18)
c.3122G>C (p.R1041P)	TKII		Classical	Karkkainen *et al*., 2000 (11)
c.3125A>G (p.N1042S)	TKII	Familial	Classical	Connell *et al*., 2009 (18)
c.3131T>C (p.L1044P)	TKII		Classical	Karkkainen *et al*., 2000 (11)
c.3145G>A (p.D1049N)	TKII	Familial	Protective factor ?	Verstraeten *et al*., 2009 (19)
c.3151G>A (p.V1051M)	TK II	Familial	Classical	Connell *et al*., 2009 (18)
c.3164A>C (p.D1055A)	TK II		Also glaucoma and learning difficulties	Connell *et al*., 2009 (18)
c.3164A>T (p.D1055V)	TK II	Familial	Classical	Yu *et al*., 2007 (16)
c.3257T>C (p.I1086T)	TK II	Familial	Classical	Ghalamkarpour *et al*., 2006 (8)
c.3316G>A (p.E1106K)	TK II		Classical (1 case diagnosed *in utero*)	Daniel-Spiegel *et al*., 2005 (18)
c.3323-3325del (p.1108delF)	TK II	Familial	Classical	Evans *et al*., 2003 (12)
c.3344A>G (p.Y1115C)	TKII	Familial	Classical	Connell *et al*., 2009 (18)
c.3341C>T (p.P1114L)	TKII		Classical	Karkkainen *et al*., 2000 (11)
c.3391G>C (p.G1131R)	TKII	Familial	Classical	Connell *et al*., 2009 (18)
c.3410C>T (p.P1137L)	TKII	Familial	Classical Classical (1 case diagnosed *in utero*)	Evans *et al*., 2003, Ghalamkarpour *et al*., 2009b (12,21)

suggesting that *FLT4/VEGFR3* mutations are strongly associated with primary valve failure in large veins. Venous reflux is, however, unlikely to contribute to the edema because the swelling manifests before the patient's adoption of upright posture (7). In the upper limbs lymphatic drainage routes and function are completely normal (7).

Milroy's disease presents either at birth or soon after it, with bilateral woody, brawny lymphedema affecting most commonly the feet up to the knees (4, 7, 23). According to a study of 71 individuals with Milroy's disease in 10 families carrying a *FLT4/VEGFR3* mutation, lymphedema was congenital in 97%, bilateral in 85% and affecting the lower limb below the knee in 94% of the cases (9). In the same study, clinical findings associated with Milroy's disease were large calibre veins (23%), upslanting toenails (10%), papillomatosis (10%), cellulitis (20%) and hydrocele (37% of male patients) (9).

Milroy's disease is rarely associated with significant complications, such as intestinal lymphangiectasia, pleural effusion, bacterial infections of the dorsal aspect of feet and toes, recurrent septic arthritis, chronic changes of the skin and intestinal lymphangiectasias (3, 6, 24). Chylothorax, chylous ascites and chylopericardium may rarely accompany the edema of the lower limbs, but they tend to appear

between 20 and 40 years of age, characterizing a variant termed as atypical Nonne-Milroy syndrome (3). There are also reports of patients with Milroy's disease who developed lymphangiosarcoma, Kaposiform hemangioepithelioma or cutaneous angiosarcoma (27-29).

The management of primary lymphedema is conservative and usually successful in most patients. Avoiding prolonged standing and elevation of the affected limb, coupled with skin care, is sufficient for the uncomplicated mild cases of lymphedema (24). Skin care is important to reduce the increased risk of cellulitis and lymphangitis (30). Maneuvers that enhance lymphatic drainage, such as compression, special exercise and manual lymphatic drainage, are also recommended (24). Complex decongestive physiotherapy (also known as comprehensive decongestive therapy) has emerged as the standard of care: it combines compression bandaging, manual lymphatic drainage (a specialized massage technique), exercise, breathing exercises, dietary measures and skin care with extensive patient education (31). Whenever medical management fails, surgical interventions are indicated as a last option: these include bypass procedures or debulking operations, to improve lymph flow and to remove lymphedematous tissue respectively (32).

Gene therapy is also investigated since it could be a powerful tool in the therapy of Milroy's disease and other forms of lymphedema as well (11, 33). Karkkainen *et al*. (11) described a knockout mouse model of Milroy's disease with an inactivating *Flt4* mutation. By using virus-mediated *VEGF-C* gene therapy, functional lymphatic vessels in these lymphedema mice were generated, suggesting that overexpression of *VEGFR3* ligands may also be used in patients, certainly in patients with Milroy's disease who also have inactivating *FLT4* mutations. In a rabit model of acquired lymphedema, overexpression of VEGF-C using a recombinant adenovirus promoted therapeutic lymphangiogenesis (33).

Milroy's disease is due to autosomal dominant mutations in the *FLT4* gene (also known as *VEGFR3*) located in 5q34-q35 (1). This gene encodes a receptor tyrosine kinase Fms-like tyrosine kinase 4 (or vascular endothelial growth factor receptor 3), which is a key factor in the development of lymphatic vessels (3, 22, 34). Thirty-seven *FLT4* mutations have been reported so far worldwide (1, 8-20) (Table I). Overall, in 75% of patients with a positive family history, and 68% of sporadic patients a *FTL4* mutation can be identified (18). All mutations reported are missense mutations or single amino acid deletions in the tyrosine kinase (TK) domains I or II of the *VEGFR3* receptor (Table I). No truncating mutations have been identified, suggesting that haploinsufficiency is not a cause of Milroy's disease. As the FLT4 receptor is a dimer with less activity in the presence of a mutated and wild-type allele than would be the case for haploinsufficiency alone, it is possible that the missense mutations lead to a "protein suicide" effect. Lack of VEGF-C ligand binding to the FLT4 receptor is not a cause of Milroy's disease, as no *FLT4* mutations were found outside the kinase domains of, and no mutations have been found in the VEGF-C ligand.

In our patient, a c.3109G>C mutation in exon 23 of the *FLT4* gene was identified. This is a known mutation which substitutes an aspartic acid into a histidine on amino acid position 1037 of the resulting protein (p.D1037H) located in the TK domain II of the receptor. This mutation has also been described in another family (18), and a family with another substitution of the same amino acid has also been described (18).

Both parents of our proband were phenotypically normal and lacked any lymphedema. Nevertheless, the c.3109G>C mutation was also present in the proband's father. Incomplete penetrance or early resolution of the edema in the first year of life leading to apparently asymptomatic patients has been described previously (8, 9).

In conclusion, we report here the identification of a c.3109G>C mutation in exon 23 of the *FLT4* gene in a patient with clinical diagnosis of Milroy's disease and his asymptomatic father. This is a known mutation which substitutes an aspartic acid into a histidine on amino acid position 1037 of the resulting protein (p.Asp1037His). Literature evidence provides data on the genetic basis, pathogenesis, clinical variability and long-term complications of Milroy's disease. A thorough genetic clinical evaluation and molecular investigation contributes to the provision of proper counseling for parents of an affected child. The optimal management of the patients requires the collaboration of geneticists, neonatologists, pediatricians, dermatologists and surgeons with awareness and knowledge of the broad spectrum of Milroy's disease.

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Received November 25, 2009
Revised March 23, 2010
Accepted March 24, 2010