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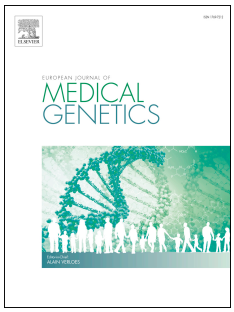

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THSD7A-Associated Membranous Nephropathy in a Patient with Neurofibromatosis Type 1

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Abstract

Target antigens in idiopathic membranous nephropathy (MN) include the phospholipase A2 receptor (PLA_2R), and in some cases, the thrombospondin type 1 domain-containing 7A (THSD7A). A notable phenomenon is the high rate of cancer (reported to be as high as 20%) in patients with THSD7A-associated MN. Neurofibromatosis type 1 (NF1) is an autosomal dominant disease caused by *NF1* gene mutation, and clinically characterized by multiple cutaneous neurofibromas and café-au-lait spots. In this article, we report a patient with NF1 who developed THSD7A-associated MN when the NF1 skin lesions deteriorated. The patient, a 62-year-old male, was referred to us for nephrotic syndrome for 6 months. Physical examination revealed multiple cutaneous nodules throughout the entire body, and the patient noted recent increase in the numbers of these skin lesions. Cutaneous nodules excisional biopsy suggested NF1 and Sanger sequencing using genomic DNA extracted from peripheral blood revealed a previously reported heterozygous frameshift *NF1* mutation (c.1541_1542delAG, p. Gln514fs). Renal biopsy revealed MN and immunohistochemistry (IHC) showed enhanced staining of THSD7A as well as PLA_2R along the glomerular basement membrane whereas the serum level of THSD7A and PLA_2Rwere both within normal range. The neurofibroma tissues were positive for THSD7A but not for PLA_2R on IHC. The patient did not respond to 6-month treatment with glucocorticosteroid and cyclophosphamide. In this

exceptional case, strong positive staining of THSD7A in both skin and renal biopsy samples, together with the temporal association between nephrotic syndrome and skin lesions and lack of treatment response, suggested the possibility that MN could be the result of immune response to THSD7A in NF1. This report may improve understanding of the mechanistic link between MN and cancer.

Key words:

Neurofibromatosis type 1, Membranous nephropathy, THSD7A

1. Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant disease (prevalence 1: 2500 to 1: 3000) caused by the mutation of the *NF1* gene that participates in the control of cell division (Lin et al., 2013). First reported by von Recklinghausen in 1882, NF1 is characterized by cutaneous neurofibromas, café-au-lait spots and axillary or inguinal freckling (Dombi et al., 2016). Patients with NF1 are at increased risk of developing malignancies and display an assortment of benign and malignant lesions including cutaneous and plexiform neurofibromas, malignant peripheral nerve sheath tumors, optic gliomas, bone abnormalities and leukemia (Boyd et al., 2013). NF1 is associated with renal artery stenosis, and in some cases with glomerular diseases, e.g., membranous nephropathy (MN), minimal change disease, focal segmental glomerulosclerosis, IgA nephropathy and IgM nephropathy (Van-Gils et al., 2010).

MN is caused by formation of immune deposits on the outer aspect of the glomerular basement membrane, which contain podocyte or planted antigens and circulating antibodies specific to those antigens (Cattran et al., 2017). Pathologically, MN is characterized by diffuse thickening of the glomerular basement membrane and widespread subepithelial deposits. The major antigen in idiopathic MN is the phospholipase A2 receptor (PLA_2R) with about 70% of idiopathic MN cases have circulation autoantibodies against PLA_2R (Beck et al., 2009). In a small but

101 significant percentage (about 2% to 9%) of the cases, the antigen responsible for the
102 immune attack in MN is thrombospondin type 1 domain-containing 7A (THSD7A)
103 (Tomas et al., 2014; Iwakura et al., 2015; Wang et al., 2017). Notably,
104 THSD7A-associated MN is strongly associated with malignant tumor. Approximately
105 20% of the patients with THSD7A-associated MN have a malignant tumor upon
106 diagnosis (Hoxha et al., 2017), in contrast to a much lower rate of 1% to 12% in the
107 overall population of MN patients (Jhaveri et al., 2013). In a previous case report,
108 surgical removal of the gallbladder cancer in a patient comorbid with
109 THSD7A-associated MN resulted in THSD7A antibody seroreversion and proteinuria
110 remission (Hoxha *et al.*, 2016). In another report of endometrial cancer comorbid
111 with THSD7A-associated MN (Hoxha et al., 2017), THSD7A was detected in the
112 metastatic lymph nodes of endometrial cancer. In this article, we report a case of
113 THSD7A-associated MN in a patient with NF1 and provide a speculation on the
114 possible mechanistic link between MN and NF1.
115

116 **2. Patient, materials and methods**

117 *2.1 Human samples*

118 This study was approved by the Institutional Review Board of Shanghai Xin Hua
119 Hospital. Peripheral blood sample was collected from the patient, whereas blood

120 samples were not available from the first-degree relatives of the patient. Written
121 informed consent was obtained from the patient.

122 *2.2 Sanger sequencing*

123 Genomic DNA was extracted from the peripheral blood sample of the patient. The
124 entire *NF1* gene coding regions and splice sites were amplified and directly
125 sequenced (primer sequences available upon request) using an ABI PRISM 3730xl
126 Genetic Analyzer (Applied Biosytems, USA). Results were analyzed based on NCBI
127 reference sequence number NM_001042492.

128 *2.3 Immnohistochemistry*

129 Paraffin-embedded sections of formalin-fixed renal biopsy tissues and cutaneous
130 nodules tissues of the NF1 patient were utilized for immunohistochemistry (IHC)
131 staining. Normal kidney sections from normal nephrectomy samples adjacent to
132 tumors were used as normal control. IHC of PLA2R, THSD7A and IgG4 on renal
133 tissues and IHC of PLA2R1, THSD7A, neurofibromin and S-100 on cutaneous
134 nodules tissues were performed as previously described (Hanai et al., 2016; Hoxha et
135 al., 2012; Tomas et al., 2014). Dilution of the primary antibodies were as follows:
136 PLA_2R (1:100, Atlas Antibodies, Sweden), THSD7A (1:50, Atlas Antibodies,
137 Sweden), IgG4 (1:300, Abcam, UK), neurofibromin (1:100, DAKO, Denmark) and
138 S-100 (1:500, Longislandbio, China). The percentage labeling index (number of

positive neurofibroma cells/total number of neurofibroma cells expressed as a percentage) was calculated.

2.4 Enzyme linked immunosorbent assay

Serum anti-PLA2R antibody was measured by Enzyme linked immunosorbent assay (ELISA) with the ELISA kit provided by EUROIMMUN, Germany [normal range 0-20 relative units (RU)/mL]. Serum anti-THSD7A antibody was measured by ELISA test with the ELISA kit provided by Jianglai Biotech, China (normal range 5.05-95.5 ng/ml) and the anti-THSD7A antibody result of the patient was also compared with that of 6 normal controls.

3. Results

3.1 Clinical characteristics of the patient

The patient is a 62-year-old male. On August 2016, he visited Renal Clinic of Xin Hua Hospital with progressive edema of both lower extremities, ascites and foamy urine. Physical examination revealed diffuse soft, cutaneous nodules of different sizes scattered on his face, trunk and extremities (Fig. 1A), which he claimed had been presented since his 30s and the number of nodules increased when edema was presented since January 2016. No café-au-lait spot, axillary freckling or any other NF1-associated clinical phenotype was observed. He had a 2+ pitting edema on both lower extremities. The patient had no family history of either kidney or skin disease.

Abnormal laboratory findings revealed heavy proteinuria (urinary protein excretion 6.8g/day), occult hematuria 2+, hypoalbuminemia 24.6g/L (total protein 43.7 g/d). Renal function was within normal range (serum creatinine, Scr 72 μmol/L). Serum anti-PLA2R antibody level was 13.57 RU/mL (normal range 0-20 RU/mL). THSD7A serum level of this patient is 19.85 ng/ml, which was not elevated compared with that in normal controls (the average THSD7A serum level was 20.19 ± 0.66 ng/ml, n=6). Laboratory testing ruled out hepatitis B/C, lupus nephritis, sarcoidosis and solid tumors or hematological malignancies other than NF1. Ophthalmologic examination, neurological examination and brain magnetic resonance imaging were normal. Cutaneous nodules excisional biopsy was performed and histology was consistent with NF1. Renal biopsy revealed features of MN with segmental endothelial and mesangial cell proliferation (Fig. 1B). IHC staining revealed enhanced staining of THSD7A (Fig.1C) and PLA_2R (Fig. 1D) along the glomerular basement membrane compared with THSD7A and PLA2R staining of normal control (Fig. 1E and Fig. 1F). The patient was also positive for IgG4 deposition in the glomeruli. IHC staining of the cutaneous nodules excisional biopsy tissues showed that the neurofibroma cells were positive for S-100 (labelling index 50%) (Fig. 2A), neurofibromin (labelling index 8%) (Fig. 2B) and THSD7A (labelling index 25%) (Fig. 2C), but negative for PLA_2R (Fig. 2D).

The patient received treatment with angiotensin-converting enzyme inhibitor (benazepril), oral prednisolone (1 mg/kg/day initially, tapering gradually to 20mg/day after 6 months) and intravenous cyclophosphamide (1.0 g/month). The patient did not respond to the treatment. Laboratory testing after 6 months revealed 24-hour proteinuria at 4.8 g, occult hematuria 2+, serum albumin 22 g/L and Scr 63 μmol/L.

3.2 Mutation analysis

Sanger sequencing in the patient revealed a previously reported heterozygous frameshift mutation c.1541_1542delAG resulting in p. Gln514fs in the *NF1* gene (Fig. 3) (Maruoka et al., 2014; Ponti et al., 2014). None of the first-degree relatives of the patient were available for sequencing.

4. Discussion

A literature search of the PubMed identified a total of 3 NF1 cases associated with membranous nephropathy, but with no molecular analysis (Tab 1). Gene sequencing in the patient in this report revealed a heterozygous frameshift mutation (c.1541_1542delAG, p.Gln514fs) in the *NF1* gene. To our knowledge, this represents the most credible case of NF1 in association with THSD7A-associated MN.

THSD7A was initially characterized as an endothelial protein that is expressed in the placental vasculature (Wang et al., 2010). In 2014, THSD7A was identified as an autoantigen in adult idiopathic MN (Tomas et al., 2014) and the prevalence of

198 THSD7A-associated MN is about 2% to 9% (Tomas et al., 2014; Iwakura et al., 2015;
199 Wang et al., 2017). Subsequent studies suggested higher rate of cancer comorbidity
200 (approximately 20%) in patients with THSD7A-associated MN (Hoxha et al., 2017).
201 Stahl et al. documented positive THSD7A in a variety of cancers including renal cell
202 carcinoma, colorectal cancer, prostate cancer and breast cancer. Notably, in patients
203 with prostate cancer, THSD7A overexpression was associated with unfavorable
204 tumor phenotype and prostate-specific antigen recurrence (Stahl et al., 2017).
205 In our NF1 patient with THSD7A-associated MN, strong positive staining of
206 THSD7A in both skin and renal biopsy samples, together with the temporal
207 association between nephrotic syndrome and skin lesions and lack of treatment
208 response, suggested the possibility that MN could be the result of immune response to
209 THSD7A in NF1. Such a notion is consistent with 2 published cases of
210 THSD7A-associated MN and malignancy, in which THSD7A was aberrantly
211 expressed in the primary gallbladder and endometrial cancer tissues and lymph-node
212 metastases (Hoxha et al., 2016; Hoxha et al., 2017). Interestingly, in the patient with
213 THSD7A-associated MN and gallbladder cancer, treatment of the primary cancer
214 resulted in THSD7A antibody seroreversion and proteinuria remission (Hoxha et al.,
215 2016). The underlying molecular mechanism for the association between
216 THSD7A-associated MN and NF1 remains obscure. One possible explanation is that
217 the immune system might have recognized THSD7A as a foreign antigen in the

218 nerufibroma cells; the resulting THSD7A antibodies then recognize THSD7A on
219 podocytes to initiate MN. However, serum THSD7A antibody, as detected by ELISA,
220 was within the normal range in our patient, as is the case for PLA_2R, which was
221 positive along the glomerular capillary wall but not detected in the serum in this
222 patient. It is plausible that alternative mechanisms (for example, the presence of
223 antibodies not recognized by the assay kits) could exist. Other possibilities might
224 include that the circulating THSD7A and PLA_2R antibodies has not yet reached a
225 level high enough or may have disappeared from the circulation, or the antibody
226 could all be bound to the antigen in the glomeruli. In the Chinese cohort of 578 MN
227 patients (Wang et al., 2017), circulating anti-THSD7A was detected in only 6 out of
228 12 (50%) patients with enhanced glomerular expression of THSD7A, whereas 120
229 out of 514 (21%) patients negative for anti-PLA_2R antibodies showed enhanced
230 expression of PLA_2R in glomeruli.
231 It is noteworthy to point out the p. Gln514fs mutation of the *NF1* gene does not
232 necessarily lead to MN; neither proteinuria nor renal dysfunction was noted in 2
233 previous reports of NF1 patients (Maruoka et al., 2014; Ponti et al., 2014). Also, the
234 patient in the current case had NF1 for over 30 years before nephrotic syndrome.
235 Spontaneous remission occurs in about 30% of idiopathic MN patients with nephrotic
236 syndrome (Polanco et al., 2010). In a previous case report of comorbid NF1 and MN
237 (antigen not determined), spontaneous remission of proteinuria was noted (Table 1;

Kokubo et al., 1993). In our case, proteinuria did not remit after 6-month therapy of oral prednisolone and intravenous cyclophosphamide. As a result, we believe that MN could either be coincident or mechanistically related depending on differing disease antigens.

In conclusion, the current case suggested the possibility that MN could be the result of immune response to THSD7A in NF1. Supporting evidence included positive THSD7A in both skin and renal biopsy samples, the temporal association between nephrotic syndrome and skin lesions, and lack of treatment response to glucocorticoids and cyclophosphamide. This report may improve understanding of the mechanistic link between MN and cancer.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgements

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Figure legends

Figure 1. A. Clinical features of the NF1 patient: multiple cutaneous neurofibroma of different sizes scattered on the neck, trunk and extremities of the patient. **Figure 1. B-Figure 1.D.** Renal histological findings of the NF1 patient: **(B)** Light microscopy showing membranous nephropathy with segmental endothelial and mesangial cell proliferation (silver staining, x40); Immunohistochemistry (IHC, x40) showed strong positive staining of THSD7A **(C)** and PLA_2R**(D)** along the glomerular basement membrane in the NF1 patient. **Figure 1. E-Figure 1.F.** IHC staining (x40) of THSD7A **(E)** and PLA_2R1**(F)** in renal biopsy tissue of normal control.

Figure 2. Microscopic findings of the cutaneous nodule excisional biopsy and the results of immunohistochemistry (x40). **A.** S-100; **B.** Neurofibromin; **C.** THSD7A; and **D.** PLA_2R1.

Figure 3. Electropherogram showing the heterozygous frameshift *NF1* mutation (c.1541_1542delAG, p. Gln514fs) in the patient **(A)** comparing to a non-carrier **(B)**.

Table 1 Neurofibromatosis type 1 associated with membranous nephropathy in the literature.

Case	Age/Gender	Family history	NF1-related features	*NF1* mutation	Renal outcome	Reference
1	62 yr/male	No family history of NF1 or kidney disease	Subcutaneous neurofibromas, no café-au-lait spots	c.1541_1542delAG, p. Gln514Argfs*43	No proteinuria remission after GC and CTX treatment	Current report
2	70 yr/female	No family history of NF1 or kidney disease	Subcutaneous neurofibromas and café-au-lait spots	No molecular analysis	N/A	Wani *et al.*, 2006
3	49 yr/male	No family history of NF1 or kidney disease	Subcutaneous neurofibromas and café-au-lait spots	No molecular analysis	N/A	Toth *et al.*, 1996
4	68 yr /male	No family history of NF1 or kidney disease	Subcutaneous neurofibromas, café-au-lait spots and axillary freckling	No molecular analysis	Spontaneous proteinuria remission	Kokubo *et al.*, 1993

CTX: cyclophosphamide; GC: glucocorticosteroid; N/A: not available; NF1: Neurofibromatosis type 1