Autoimmune disease and multiple autoantibodies in 42 patients with RASopathies

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The association of RASopathies [Noonan syndrome (NS) and Noonan-related syndromes] and autoimmune disorders has been reported sporadically. However, a concomitant evaluation of autoimmune diseases and an assessment of multiple autoantibodies in a large population of patients with molecularly confirmed RASopathy have not been performed. The clinical and laboratory features were analyzed in 42 RASopathy patients, the majority of whom had NS and five individuals had Noonan-related disorders. The following autoantibodies were measured: Anti-nuclear antibodies, anti-double stranded DNA, anti-SS-A/Ro, anti-SS-B/La, anti-Sm, anti-RNP, anti-Scl-70, anti-Jo-1, anti-ribosomal P, IgG and IgM anticardiolipin (aCL), thyroid, anti-smooth muscle, anti-endomyosal (AE), anti-liver cytosolic protein type 1 (LC1), anti-parietal cell (APC), anti-mitochondrial (AM) antibodies, anti-liver-kidney microsome type 1 antibodies (LKM-1), and lupus anticoagulant. Six patients (14%) fulfilled the clinical criteria for autoimmune diseases [systemic lupus erythematosus, polyendocrinopathy, primary antiphospholipid syndrome (PAPS), autoimmune hepatitis, vitiligo, and autoimmune thyroiditis]. Autoimmune antibodies were observed in 52% of the patients. Remarkably, three (7%) of the patients had specific gastrointestinal and liver autoantibodies without clinical findings. Autoimmune diseases and autoantibodies were frequently present in patients with RASopathies. Until a final conclusion of the real incidence of autoimmunity in Rasopathy is drawn, the physicians should be alerted to the possibility of this association and the need for a fast diagnosis, proper referral to a specialist and ultimately, adequate treatment.
cardiofaciocutaneous syndrome (CFC, OMIM: 115150), NS with multiple lentigines (NSML, OMIM: 151100), and NS with loose anagen hair (NSLAH, OMIM: 607721). Although each RASopathy presents characteristic phenotypic features, they all share common traits, such as craniofacial dysmorphisms, short stature, cardiac malformations, variable cognitive delay, and an increased risk of cancer development [Romano et al., 2010; Tartaglia et al., 2011]. Anecdotal reports described the association of RASopathies and autoimmune disorders, such as thyroiditis, vasculitis, celiac disease, vitiligo, and systemic lupus erythematosus (SLE) [Chaves-Barbalo and Hayles, 1966; Venerus and Aarskog, 1973; Berberich and Hall, 1976; Sharland et al., 1992; Martin et al., 2001; Amoroso et al., 2003; Alanay et al., 2004; Yamashita et al., 2004; Lopez-Rangel et al., 2005]. However, to the best of our knowledge, the concomitant evaluation of autoimmunity diseases and multiple autoantibody assessments in a large population of RASopathy patients has not been performed.

Therefore, the aim of this study was to determine the frequency of clinical and laboratory features associated with autoimmunity and inflammation in a cohort of NS and Noonan-related-disorder patients with proven mutations in RAS/MAPK pathway genes.

**MATERIALS AND METHODS**

A prospective study including 42 patients was performed at the Outpatient Clinic of the Genetics Unit of the Children's Hospital, Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo. Clinical criteria were used to classify patients as affected by NS, NSML, and CFC syndrome [Voron et al., 1976; van der Burgt et al., 1994; Kavamura et al., 2002; Roberts et al., 2006]. One patient presenting with *PTPN11* p.T468M, which is usually associated with NSML, was classified as NS because he has lacked lentigines by age 19 years. On the other hand, this patient did present other pigmented skin abnormalities, such as café-au-lait spots and hypertrophic cardiomyopathy, both features frequently associated with this particular *PTPN11* mutation. A diagnosis of NSLAH was determined by slow growing, fine hair and the presence of a mutation (p.S2G) in one patient who was negative for previously hypertrophic cardiomyopathy, both features frequently associated with NSLAH (OMIM: 607721).

**PART A**
RESULTS

Patients’ age ranged from four to 57 years, with a mean of 19 years and a median of 17 years. Gender was equally distributed, with 22 females and 20 males. The cohort (Table I) comprised 37 NS patients, two CFC, two NSML, and one patient with NSLAH. The majority of the diagnoses (35 patients, 83%) were considered sporadic due to a de novo mutation, while familiar aggregation was found in seven patients (one family of three, and two families with two individuals). Three patients had already been diagnosed with autoimmune thyroiditis, and one of them had concomitant celiac disease. One additional NS patient, with a diagnosis of autoimmune hepatitis, could not undergo our complete rheumatologic investigation for logistical reasons and is not included in Table I.

A total of 22 patients (52%) presented with at least one autoantibody described below. Five presented with the concomitant occurrence of two autoantibodies.

The presence of ANA was detected in five patients (titer 1:80 to 1:320), anti-TG in four (50–699 U/ml), anti-TPO in two (63 and 1,416 U/ml), lupus anticoagulant in three, and anti-SMA in two patients (titer 1:160 vascular/glomerular and 1:80 glomerular).

APC antibodies (titer 1:40), anti-endomysium antibodies (titer 1:80), anti-mitochondria antibodies (titer 1:160), RF (32 IU/ml), and cryoglobulinemia (1.43 μg/ml) were found in one patient each (Table I).

C-reactive protein levels and the erythrocyte sedimentation rate (both inflammatory markers) were slightly elevated in one patient each. Although TSH was elevated in nine patients (5.27–137 mU/ml, median of 6.5), no new case of overt hypothyroidism was noted. Platelet count was normal in all, and no patients presented evidence of hemolytic anemia, although four had low-grade iron-deficiency anemia. Based on these results, no further hematological investigation for autoimmunity was performed.

Organ-specific antibodies were found in three patients not fulfilling clinical criteria for the diagnosis of an autoimmune disease. We observed a 9-year-old NS patient with subclinical hypothyroidism who was positive for APC antibodies, a marker of autoimmune gastritis and consequent pernicious anemia, but the patient presented no evidence of a B12-deficiency. A 10-year-old and a 27-year-old male NS patient presented positive anti-smooth muscle and/or anti-mitochondria antibodies without evidence of autoimmune hepatitis.

<table>
<thead>
<tr>
<th>RASopathies</th>
<th>Gene</th>
<th>Mutation</th>
<th>Autoimmune marker</th>
<th>Disease</th>
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<tbody>
<tr>
<td></td>
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<td>p.G60A</td>
<td>1/28</td>
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<td>p.i282V</td>
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<td>p.N308D</td>
<td>9/28</td>
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<td>p.N308S</td>
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<td>p.S2G</td>
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<td>CI [1/1]</td>
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</table>

*Table excludes an NS patient with autoimmune hepatitis (discussed separately). Autoimmune marker: lupus anticoagulant, LA; thyroid antibodies: anti-TG and anti-TPO; anti-smooth muscle, ASM; anti-mitochondria, AM; circulating immunocomplex, CI; cryoglobulinemia, Cryo; rheumatoid factor, RF; anti-parietal cell, APC; anti-endomysium, AE. Disease: subclinical hypothyroidism, SH; systemic lupus erythematosus, SLE; overt hypothyroidism, OH; celiac disease, CD; primary antiphospholipid syndrome, PAPS; vitiligo, Vi.
An autoimmune disease was diagnosed in six patients (14%), described below.

A 54-year-old NS patient with a positive RF test, but no other symptoms suggesting rheumatoid arthritis, presented with characteristic vitiligo skin lesions.

A 32-year-old female (PTPN11 p.F285S) with a history of treated autoimmune hypothyroidism complained of photosensitivity, arthralgia, and excessive hair loss. Active arthritis and livedo reticularis were found during her physical examination. Laboratory screening indicated a high ANA titer (1:320, homogeneous), leucopenia (1,970 × 1,000/mm³), and lymphopenia (759 × 1,000/mm³). The patient’s findings fulfill the revised diagnostic criteria for SLE [Hochberg, 1997].

No specific SLE autoantibody (such as anti-P, anti-Sm, and anti-dsDNA) was found in the studied population, including the NS patient with SLE discussed above.

An 8-year-old female (PTPN11 p.Y63C) presenting with autoimmune thyroiditis and several rheumatic complaints (such as arthralgia, myalgia, and gastrointestinal symptoms) also tested positive on an anti-endomysium antibody test (titer 1:80) and her colonoscopy revealed mucosal atrophy. Her symptoms improved following the initiation of a gluten-free diet. These findings fulfill the diagnostic criteria for celiac disease [Catassi and Fasano, 2010]. This is the only patient in our cohort with a family history of autoimmunity: Her mother had been diagnosed with autoimmune thyroiditis and does not carry the PTPN11 mutation.

An 8-year-old female with NS (PTPN11 p.D106A) had a previous history of venous thrombosis following a surgical procedure. The presence of lupus anticoagulant and a previous thrombotic event fulfill the diagnostic criteria for antiphospholipid syndrome [Miyakis et al., 2006].

Subclinical hypothyroidism (high TSH and normal free-T4) was discovered in seven patients (17%) without thyroid antibodies. Three of these patients carried a mutation in PTPN11 (p.N308S). Overt hypothyroidism requiring hormone replacement and high levels of thyroid antibodies was observed in three patients (7%). The diagnosis of this endocrinopathy was established at age 7, (the patient described above with associated polyendocrinopathy), 21, and 24 years (the patient described with SLE). We also observed thyroid antibodies in three participants (7%) whose TSH and free-T4 levels were within the normal ranges.

Included in the study was a 19-year-old male affected by NSML (PTPN11 p.Y279C). The patient began complaining of abdominal pain at age 10 years. At the initial evaluation, an ultrasound study discovered hepatosplenomegaly and high levels of liver enzymes (AST, ALT, alkaline phosphatase, GGT). In addition, inflammatory markers (C-reactive protein and hemosedimentation rate) were observed. Alpha1-antitrypsin, ceruloplasmin, serological tests for hepatitis B and C were all within the normal range. Urine and serum levels of copper were also within the normal range. The patient was diagnosed with autoimmune hepatitis based on positive levels of anti-SMA (two separate measurements of titers of 1:40 and 1:80) and a liver biopsy [Alvarez et al., 1999]. The biopsy revealed fibrosis, disorganization of the liver architecture, and inflammatory infiltrate. The patient’s symptoms and liver markers improved after administration of azathioprine and corticosteroids; however, he developed diabetes mellitus following an episode of spontaneous bacterial peritonitis and required insulin therapy.

No significant statistical difference was noted among the different groups stratified by their respective altered gene, either when considering the presence of autoantibodies or autoimmune diseases.

DISCUSSION

We present the first prospective study examining autoimmune and inflammatory markers in patients affected by RASopathies confirmed by molecular analysis. The results indicate a high frequency of autoantibodies and autoimmune diseases; thus, it is important to consider autoimmune diseases in children and adults with RASopathies.

Most of the study participants were affected by NS with only five presenting NS-related disorders. Therefore, our findings cannot be considered representative for Noonan-related syndromes.

In our study, the markers most frequently found were ANA, which is a nonspecific antibody with limited value as a diagnostic tool by itself, and anti-thyroid, an organ-specific antibody associated with the most common autoimmune disorder, thyroiditis.

The frequency of autoimmune diseases in the general population ranges from 5 to 8% in the USA [National Institutes of Health Autoimmune Disease Coordinating Committee Report, 2002]. In the present study, autoimmune disorders were noted in 14% of the patients (two-to-threefold increased risk), and included autoimmune thyroiditis, SLE, polyendocrinopathy (association of autoimmune thyroiditis and celiac disease), antiphospholipid syndrome, vitiligo, and autoimmune hepatitis. All diseases were observed in patients harboring PTPN11 mutations. Except for autoimmune hepatitis, all autoimmune diseases found in our cohort have been previously described in NS, especially SLE and thyroid disorders. The yearly incidence of autoimmune hepatitis has been estimated at approximately one to two cases per 100,000 inhabitants for females in Europe, with a fivefold lower incidence in men [Primo et al., 2004]. This is the first association of autoimmune hepatitis in a boy with RASopathy, which is likely very rare.

In literature reports of SLE associated with NS [Martin et al., 2001; Amoroso et al., 2003; Alanay et al., 2004; Mustelin, 2006; Lisbona et al., 2009; Leventopoulos et al., 2010] authors emphasize the young age of onset. Interestingly, linkage analysis on SLE susceptibility loci has been reported for different loci, including a locus at 12q24.1. This locus also contains PTPN11, the main gene involved in NS [Lopez-Rangel et al., 2005; Lisbona et al., 2009]. The linkage of a susceptibility gene for SLE to an area that is also directly involved in the occurrence of RASopathy strengthens the hypothesis of a common origin, although no definitive study has yet been performed.

The prevalence of subclinical hypothyroidism, overt hypothyroidism, and thyroid antibodies in the normal Brazilian population has been estimated to be 12.7, 5.4, and 16.2%, respectively, and is slightly lower than the rates observed in our cohort [Benseñor et al., 2011]. The presence of thyroid disease in NS has been described previously, but its prevalence has been a matter of debate. Two studies [Vesterhus and Aarskog, 1973; Sharland et al., 1992] described a high prevalence of hypothyroidism (26 and 20%, respectively).
respectively) and thyroid antibodies (30 and 60%, respectively). However, another study [Svensson et al., 2003] did not find a significant difference between NS patients and the typical population, similar to our findings.

The lupus anticoagulant antibody has been shown to be associated with an increased risk of thrombotic events [Espinosa and Cervera, 2010]. Three patients were positive for this marker in our study. One fulfilled the criteria for primary antiphospholipid syndrome (PAPS), which was identified previously in a 12-year-old NS patient with Moyamoya-like vascular changes [Yamashita et al., 2004]. The occurrence of pro-thrombotic events in RASopathy broadens the spectrum of findings within coagulation defects, usually manifested by bleeding diathesis [Romano et al., 2010].

Some autoantibodies were discovered in patients without any other sign of disease activity. Close surveillance will be maintained to determine the implication of these markers in the development of future autoimmune disorders, especially the patients with organ-specific antibodies as recently described by our group in regards to juvenile SLE and juvenile dermatomyositis [Aikawa et al., 2011]. Interestingly, the frequency of organ-specific antibodies (associated with celiac disease, autoimmune gastritis, and autoimmune hepatitis) in the present study was similar to those found by Aikawa et al. [2011] in the previously mentioned autoimmune-prototype diseases.

Several pieces of evidence point to the role of RAS/MAPK signaling pathway in general immunity. RAS is a GTP-binding protein that plays multiple roles in cell activation, including proliferative and inflammatory responses necessary for the maintenance of immune tolerance. Animal and human immunologic models have shown that the failure of this complex signaling pathway may lead to autoimmunity [Stone, 2006; Mor et al., 2007]. The product of the PTEN (SHP-2) is important for the maintenance of resting lymphocytes and regulation of the transcription factor NF-kB, which plays a role in antibody production and activation of natural killer (NK) cells [Iivanainen et al., 1990; Mustelin et al., 2005; Mustelin, 2006; Vang et al., 2007].

These studies highlight the involvement of different genes of the RAS/MAPK pathway in immunity. In our cohort, this involvement was corroborated by the presence of autoantibodies in patients harboring mutations in PTEN, SOS1, BRAF, and SHOC2.

A limitation of our study is the lack of a control group. In addition, there is no comprehensive study in the literature that has determined the exact frequency of the autoimmune markers or autoimmune diseases in the general population. Without such a baseline, any comparisons of the frequency of these parameters between patients with RASopathy and the general population are compromised.

In this study, autoantibodies and autoimmune diseases were frequently observed in RASopathy patients, suggesting an association. Although this fact may have implications in the follow-up of these patients, it is premature to recommend routine screening for autoimmunity in asymptomatic RASopathy patients.

In our cohort, all patients with autoimmune disorders were systematically referred to specialists (rheumatologist, endocrinologist, gastroenterologist, or dermatologist). Patients with organ-specific autoantibodies are being monitored for possible complications in our outpatient Genetics clinic.

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REFERENCES


