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Integrated genetic diagnosis of Neurofibromatosis type 1 (NF1) and molecular characterization of one case of compound heterozygosity

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INTRODUCTION

Neurofibromatoses

Historically the neurofibromatosis type 1 (NF1), type 2 (NF2) and schwannomatosis were referred with the common terms of Neurofibromatosis. Neurofibromatoses are a group of conditions that predispose to tumors of the nervous system and abnormal skin pigmentation. Each type is defined by the presence/absence of café au lait (CAL) spots and skinfold freckling, what kind of peripheral nerve tumor develops (neurofibromas vs. schwannomas) and other features, particularly in the eye, specific to each form. The neurofibromatoses, predisposing to multiple tumors of the peripheral nervous system, are often considered classical tumor suppressor diseases. Longitudinal care for individuals with neurofibromatosis aims at the early detection and symptomatic treatment of complications as they occur [1].

Very much has been elucidated about the complex molecular mechanism leading to these diseases. In the last two decades of the last century, the modern methods of genetic research showed that NF1 and NF2 had distinct clinical and genetic features. More recently, a subtype of NF has been defined [2-4] as has schwannomatosis [5] the NF2 related condition.

Ricciardi first recognized the importance of clear distinction of the different types of NF1 and proposed a numerical classification system based upon the presence/absence of CAL spots, skinfold freckling, neurofibromas/schwannomas and Lisch nodules (Table 1)[6]. This classification formed the basis for the nowadays-recognized classification and diagnostic criteria developed in 1988 by National Institutes of Health (NIH) (Table 1) [7].

Neurofibromatosis type 1	**Von Recklinghausen disease**
Neurofibromatosis type 2	Bilateral acoustic neurofibromatosis
Neurofibromatosis type 3	Mixed neurofibromatosis
Neurofibromatosis type 4	Variant neurofibromatosis
Neurofibromatosis type 5	Segmental neurofibromatosis
Neurofibromatosis type 6	CALs-only
Neurofibromatosis type 7	Late onset
Others not specificated	

Table 1. *Neurofibromatosis Classification by Ricciardi - Eichner, 1982* [6]

Neurofibromatosis type 1

A German pathologist, Friedrich Daniel von Recklinghausen, published in 1882 the first precise clinical and pathological characterization of neurofibromatosis type 1. In his honor NF1 is also common called as von Recklinghausen syndrome[8].

NF1 is one of the most common human autosomal dominant

disorders, with an estimated incidence of 1:3500 live births. Approximately one-half of the cases are familiar (inherited); the remainders are the result of de novo (sporadic) mutations. It is clinically characterized by CALs, axillary and/or inguinal freckling, cutaneous and plexiform neurofibromas (NFs), Lisch nodules of the iris, optic gliomas, specific bone lesions and increased risk of malignant tumors. Clinical diagnosis is based on NIH NF1 criteria [7, 9]. The condition is fully penetrant and has a highly variable expression, even within the same family. NF1 is caused by mutations in neurofibromin gene *NF1* (NM_000267.2). The *NF1* gene is located on human chromosome 17q11.2 and was identified by positional cloning in 1990 [10, 11].

Clinical Characterization

Neurofibromatosis type 1 would be better defined as "neuro-cardio-facial-cutaneous (NFNC) syndrome", along with Noonan, LEOPARD, cardio-facio-cutaneous (CFC) and Costello syndrome and recently also Legius syndrome. These conditions all share a variable degree of learning difficulty, cardiac defect, overlapping facial dysmorphism, short stature, macrocephaly and skin abnormalities. All these syndromes were also called "Rasopathies" because they result from germline mutations in the evolutionarily conserved Ras-MAPKinase pathway [12-14].

Neurofibromatosis type 1 is diagnosed clinically, in which an individual must fulfill at least 2 clinical criteria [7, 15] (Figure 1):

- Six or more cafè-au-lait macules of over 5mm in greatest diameter in prepubertals and over 15mm in greatest diameter in postpubertal individuals
- Two or more neurofibromas of any type or one plexiform neurofibroma
- Freckiling in the axillary or inguinal regions
- Optic glioma
- Two or more Lisch nodules (iris hamartomas)
- A distinctive osseous lesion such as sphenoid dysplasia or thinning of the ling bone cortex with or without pseudarthrosis
- A first-degree relative (parent, sibling, or offspring) with NF1 by the above criteria

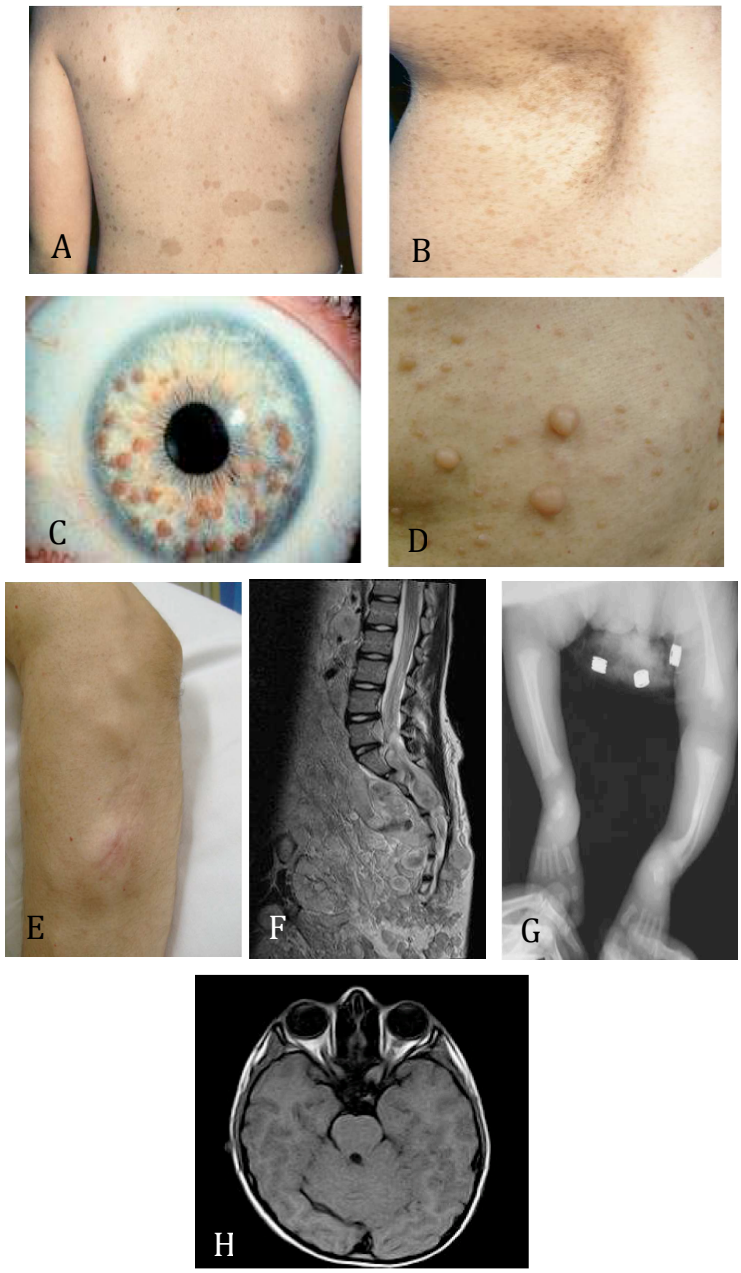

Figure 1. *NF1's typical signs: A) Café-au-lait macules; B) Freckiling; C) Lisch nodules; D) Cutaneous neurofibromas; E) Subcutaneous neurofibromas; F) Plexiform neurofibromas; G) Tibial dysplasia; H) Optic glioma (left).*

These clinical criteria are both highly specific and highly sensitive in adults with NF1[16]. In children, the diagnosis can be more problematic. Only approximately one half of the children with NF1 and no family history of NF1 meet the criteria for diagnosis by the age of 1 year, but almost all do by the age of 8 years because many features of NF1 increase in frequency with age (Figure 2). [17-19]. Children who have inherited NF1 from an affected parent can usually be identified within the first year of life because diagnosis requires just one feature in addition to a positive family history. This feature is usually multiple CALs, which develop in infancy in >95% of individuals with NF1. A definite diagnosis of NF1 can be made in most of these children by the age of 4 years using the NIH criteria [17].

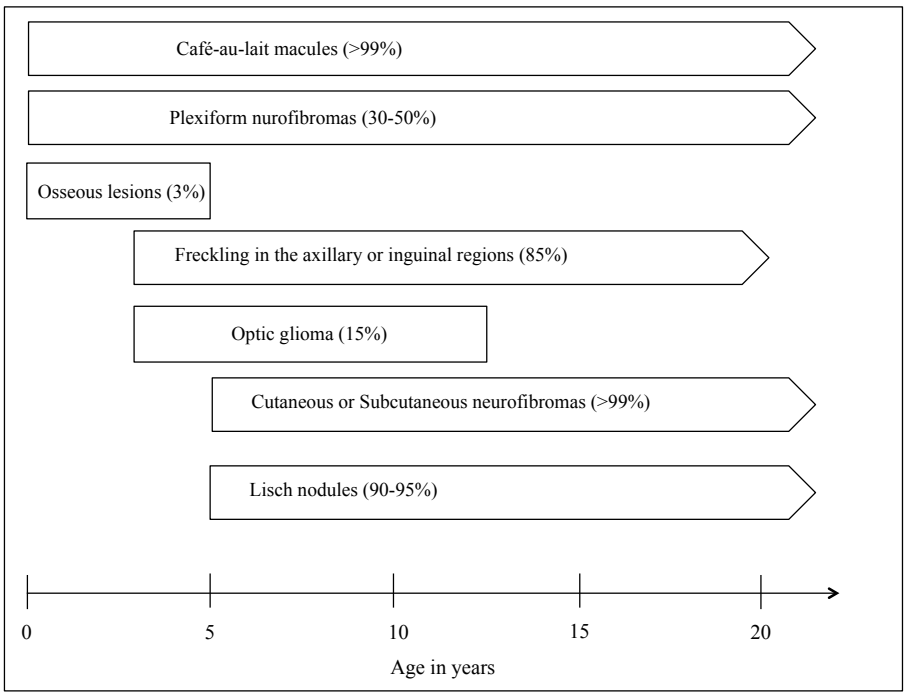

Figure 2. *Frequency and Age of onset of NF1's prymary signs (modified from PDTA of Regione Lombardia).*

Genetic Testing

At the moment genetic testing is not include in NIH criteria to define NF1 condition. However it is very helpful in confirming diagnosis in proband and in establishing diagnosis in patients with a NF1's subtype phenotype whom do not have a fulfill the NIH diagnostic criteria:

- Confirmatory diagnostic testing is indicated for individuals in whom NF1 is suspected but who do not fulfill the NIH diagnostic criteria. This is rarely necessary after early childhood.
- **Prenatal diagnosis and pre-implantation genetic diagnosis (PGD)** for at-risk pregnancies require prior identification of the disease-causing mutation in the family.

- Molecular testing for NF1 is infrequently indicated clinically but may be useful in a young child with a serious tumor (e.g., optic glioma) in whom establishing a diagnosis of NF1 would affect management.
- **A multi-step detection protocol** that identifies more than 95% of pathogenic *NF1* mutations in individuals fulfilling the NIH diagnostic criteria is available [20].
- **A mild variant associated with a 3-bp in-frame deletion of exon 17 (c.2970-2972delAAT)** (NF Consortium nomenclature, exon 22 in NCBI nomenclature), in which neurofibromas are rare and multiple café-au-lait spots may be the only apparent manifestation (see "NF1 subtype") [3].
- **Familial spinal neurofibromatosis,** in which multiple spinal neurofibromas but few, if any, cutaneous manifestations of NF1 occur. Despite the name, this condition may also occur in sporadic

cases. (see "NF1 subtype") [21].

- A similar disorder in a patient with multiple spinal ganglioneuromas (rather than neurofibromas) and multiple subcutaneous tumors [22].
- A man with optic glioma but no other diagnostic features of NF1 [23].
- A child with encephalocraniocutaneous lipomatosis [24].

Clinical features

Cafe´-au-lait macules (CALs) occur in nearly all affected individuals and often are present at birth and increase in number during the first few years of life. CALs are >5 mm in greatest diameter in prepubertal individuals and >15 mm in greatest diameter in post pubertal individuals.

Freckling occurs mostly in regions of skin apposition, especially the axillary and groin areas. It develops in almost 90% of patients, usually by the age of 7 years.

Lisch nodules are innocuous iris hamartomas. They are not present at birth but can be found in >90% patients with NF1 aged 16 years or older.

Neurofibromas or **plexiform neurofibroma**: Neurofibromas are benign peripheral nerve sheath tumors that are comprised of a mixture of Schwann cells, fibroblasts, and mast cells. The Schwann cells may be abnormal in NF1 patients, and they can have angiogenic and invasive properties in plexiform neurofibromas [25].

Cutaneous neurofibromas

Cutaneous neurofibromas consist of soft, fleshy, sessile or pedunculated tumors that appear similar to skin tags [26]. Neurofibroma formation is most common in the skin but may affect virtually any organ in the body. Discrete cutaneous and subcutaneous neurofibromas are uncommon in early childhood. In adults with NF1, numerous cutaneous neurofibromas are usually present, but the total number varies from a few too many thousands. Cutaneous neurofibromas are benign and do not carry an increased risk of developing malignant transformation. However, they often represent a major cosmetic problem in adults [27].

Plexiform neurofibromas

Approximately one half of people with NF1 have plexiform neurofibromas, but most are internal and not suspected clinically. Plexiform neurofibromas can grow from nerves in the skin or from more internal nerve bundles, and can be very large. As a result of their extent and location, some plexiform neurofibromas cause disfigurement and may compromise function. There are two different types of plexiform neurofibroma, deep nodular neurofibroma (also called nodular plexiform neurofibroma) and diffuse plexiform neurofibroma. Both types can transform into malignant peripheral nerve sheath tumors (MPNSTs) [27].

Optic pathway glioma: is the most frequent brain tumor in NF1 children. Approximately 15% of patients with NF1 develop optic pathway gliomas that are apparent on magnetic resonance imaging (MRI) before 6 years, but most are asymptomatic and remain so

throughout life [28]. They can arise anywhere along the anterior visual pathway to the optic radiations and involve the optic nerves, chiasm, and postchiasmal optic tracts. Symptomatic optic pathway gliomas in NF1 are frequently stable for many years or only very slowly progressive; some of these tumors spontaneously regress [29].

Furthermore brain tumors, which are usually gliomas of the brain stem or cerebellum, occur much more frequently than expected in people with NF1, especially in children and young adults. Brain tumors usually follow a less aggressive course in people with NF1 than in other individuals [30, 31].

Bone abnormalities in NF1 include pseudoarthrosis and bone dysplasia, as well as short stature, scoliosis, and osteoporosis. Scoliosis has been reported in 10% to 26% of individuals affected with NF1 in various clinic-based series. Long bone dysplasia, most often involving the tibia, occur in 1% to 4% of children with NF1 in clinic-based series. In infants with tibial dysplasia, the bone is usually bowed in an anterolateral direction and is subject to pathologic fracture.

Individuals with NF1 tend to be below average in height and above average in head circumference for age [27].

Neurobehavioral abnormalities

Neurologic abnormalities — Neurologic disorders include cognitive deficits, learning disabilities, and seizures. Gross and fine motor developmental delays are also seen. Macrocephaly is a common feature. Headaches occur frequently among individuals with NF1, and

hydrocephalus or seizures are seen occasionally [27].

Most individuals with NF1 have normal intelligence, but learning disabilities occur in 50% to 75%.

Children with NF1 often have poorer social skills and other personality, behavioral, and quality of life differences when compared with children without NF1 [27].

Unidentified bright objects (UBOs), which are sometimes called "T2 hyperintensities" or "focal areas of signal intensity", can be visualized on T2-weighted MRI of the brain in at least 60% of children with NF1, but the clinical significance is uncertain [32].

Tumors associated to NF1

People with NF1 have an increased relative risk of tumors occurrence. Mutations in the *NF1* gene result in loss of a functional protein causing the wide spectrum of clinical findings including NF1-associated tumors. Neurofibromin plays a role as a tumor suppressor gene (see paragraph "NF1 protein").

Malignant peripheral nerve sheath tumors (MPNSTs) are the most frequent malignant neoplasms associated with NF1, occurring sometime in the life of >10% of affected individuals. Two to 3% of people with NF1 develop a diffuse polyneuropathy that may be associated with multiple nerve root neurofibromas and a high risk of developing MPNSTs [33]. These malignancies tend to develop at a much younger age and have a poorer prognosis for survival in people with NF1 than in the general population. High-grade MPNSTs usually arise in patients with NF1 in their 20s or 30s [34].

Leukemia, especially juvenile myelomonocytic leukemia and myelodysplastic syndromes, is infrequent in children with NF1 but much more common than in children without NF1. In one population-based study, women with NF1 appeared to have a 5-fold increased risk of developing breast cancer before the age of 50 years and a 3.5-fold increased risk of developing breast cancer overall [35]. Gastrointestinal stromal tumors are also unusually frequent in people with NF1. NF1-associated and sporadic gastrointestinal stromal tumors appear to have different molecular pathogenesis, which has important implications in terms of therapy [36].

NF1 Complications: These features in NF1 patients occur more frequently than general population. New associations with NF1 are still being recognized.

Vasculopathy

A characteristic NF1 vasculopathy can cause arterial stenosis, occlusion, aneurysm, pseudoaneurysm, rupture, or arteriovenous fistula formation. NF1 vasculopathy involving the arteries of the heart or brain or other major arteries can have serious or even fatal consequences.

The prevalence of hypertension is more common in people with NF1 than in the general population and may develop at any age. Moreover, valvular pulmonic stenosis and coarctation of the aorta are more common in individuals with NF1 than in the general population [27].

Involvement of the endocrine

Delayed puberty is also common, but the reason it occurs is unknown [37].

NF1 may have lower than expected serum 25-hydroxyvitamin D concentrations, elevated serum parathyroid hormone levels, and evidence of increased bone resorption [38].

Disease feature	Frequency (%)[a]	Age of presentation
Café au lait (CAL) spots	>99	0–2 years
Skinfold freckling	67	3–5 years
Dermal neurofibromas	>95 of adults	≥7 years, usually postpubertal
Nodular neurofibromas	48[b]	Occasionally <10 years, usually start developing from teens
Plexiform neurofibromas		
All lesions	30.0	0–18 years
Large lesions of head and neck	1.2	0–1 year
Limbs/trunk lesions associated with hypertrophy	5.8	0–5 years
Lisch nodules	90–95	≥3 years
Macrocephaly	45	Birth
Short stature	31.5	Birth
Intellectual handicap		
Severe	0.8	0–5 years
Moderate	2.4	0–5 years
Minimal/learning difficulties	29.8	0–5 years
Epilepsy		
No known cause	4.4	Lifelong
Secondary to disease complications	2.2	Lifelong
Hypsarrhythmia	1.5	0–5 years
CNS tumors		
Optic glioma	1.5[c]	0–20
Other CNS tumors	1.5	Lifelong
Spinal neurofibromas	1.5	Lifelong
Aqueductal stenosis	1.5	Lifelong, usually <30 years
Malignancy		
MPNST	1.5[d]	Lifelong
Rhabdomyosarcoma	1.5	0–5 years

Orthopedic		
Scoliosis, requiring surgery	4.4	0–18 years
Scoliosis, less severe	5.2	0–18 years
Long bone dysplasia	3.7	0–2 years
Vertebral scalloping	10.0[e]	Lifelong
Gastrointestinal stromal tumours	2.2	Lifelong
Renal artery stenosis	1.5	Lifelong
Pheochromocytoma	0.7	≥10 years
Duodenal carcinoid	1.5	≥10 years
Juvenile xanthogranuloma	0.7	0–1 year
Congenital glaucoma	0.7	0–1 year
Sphenoid wing dysplasia	<1	Congenital
Lateral meningocele	<1	Lifelong
Juvenile myelomonocytic leukemia	<1	0–18 years
Cerebrovascular disease	<1	Childhood
Glomus tumours of nailbed	<1	Usually in adults
Breast cancer in women	Relative risk ×3 for those <50 years	

Figure 3. *Frequency and Age of onset of NF1's primary and secondary signs*[1].

Clinical NF1 subtypes

The various forms of NF have quite different implications for patients in terms of management and genetic counselling.

Segmental or localized NF1

Mosaic or segmental NF1 is the most frequent NF1 subtype: it is used to describe the patients with disease features limited to one or more body segments. Ruggeri and Huson [39] estimated disease prevalence to be between 1 in 36.000 to 40.000 individuals in general population. Most patients are asymptomatic. In the majority of patients the area involved is unilateral and varies in size from narrow strip to one quadrant and occasionally one half of the body [1]. Some patients have

more than one segment involved on both sides of the midline, either in a symmetrical or asymmetrical arrangement. Within the affected area the patients either have NF1-related pigmentary changes, neurofibromas alone, or both. The importance of recognizing this group is for their different natural history and because they have much lower recurrence risks in offspring's. However parents with gonosomal mosaics could have children with full-blown NF1 [40]. The phenotype reflects the embryonic timing and, therefore, the neural crest-derived cell types involved in the somatic mutation.

The CAL-only phenotype

This term is used to describe multi-generational families with multiple CAL spots as their main disease feature sometimes associated with axillary freckling. Two recent studies have further elucidates the genetic basis of this phenotype:

1- NF1 exon 17 3bp inframe deletion. Upadhyaya et al. [3] recently reported 21 unrelated probands (14 familiar and 7 sporadic) with the same c.2970_2972delAAT (p.990delM) mutation but no cutaneous neurofibromas and no clinical plexiform neurofibromas. Of the total cohort (n=47), onl7 30 had axillary freckling. There was also a different frequency of complications, with a much lower frequency of learning problems, macrocephaly and short stature; a similar frequency of scoliosis butt with an increased frequency of pulmonary stenosis. The main importance of the phenotype was the lack of dermal neurofibromas in adult patients.

2- Legius syndrome (formally called NF1-like syndrome) is a relatively newly described autosomal dominant RASopathy. Brems et al. [4] reported mutations in the *SPRED1* gene on chromosome 15. In a large NF1 clinic they identified five familes with CAL spots, axillary freckling, macrocephaly and Noonan like facies in some individuals. No neurofibromas or Lisch nodules were present. On the other hand, learning problems, ADHD and pectus excavatum were diagnosed in several individuals. When *NF1* mutations were not identified, they did linkage studies in the two largest families and mapped the locus to chromosome 15. Legius syndrome is caused by heterozygous inactivating mutations in SPRED1 [4], a negative regulator of Ras, by inhibiting phosphorylation of Raf [41], and a causative dysregulated signaling of the Ras/MAPK pathway.

Watson Syndrome

Watson [42] described autosomal dominant inheritance of pulmonary stenosis, multiple CALs and intelligence at the lower end of the normal range. At that time pulmonary stenosis was not recognized as an NF1 complication and few similar families have since been reported [43]. Many people with this condition also have a larger than average head size (macrocephaly) and Lisch nodules. While mutations in the NF1 gene have been found and segregated in families with Watson syndrome, the exact cause of this condition is unknown. *NF1* mutation alone is not sufficient to explain this distinctive phenotype.

Neurofibromatosis-Noonan Syndrome (NFNS)

A reasonable number of patients with NF1 do have facial features that overlap with mild Noonan syndrome, with mild ptosis and hypertelorism, down-slating palpebral fissures, and posteriorly rotatedears [44, 45]. These patients have *nf1* gene mutations [46]. Given the recent findings of mutations in other components of the Ras-MAPK pathway in Noonan and other syndromes with features overlapping NF1 and Noonan's, one possibility to explain the variable NFNS phenotype is the interaction with functional polymorphisms in other genes in the pathway [1].

Spinal Neurofibromatosis

Patients with NF1 can develop neurofibromas on the dorsal spinal roots, either as a single entity or at several consecutive levels as part of a plexiform neurofibroma. There is a subset of NF1 patient where the spinal root tumors are the principle feature. Families with this consistent phenotype have been reported [47-49]. Pulst et al [49] reported one family which did not map to the NF1 locus. Messian et al. [50] have recently described 22 adults with the phenotype with few NF1 pigmentary features and absence of dermal neurofibromas but with multiple spinal neurofibromas with or without involvement of peripheral nerves. They identified NF1 mutation in 18/22 of the cohort, suggesting there may be genetic heterogeneity. They found an over-representation of missense and splice mutation then general NF1 mutation spectrum. These findings may point towards a different requirement for dermal versus spinal root neurofibromas.

Genotype/Phenotype correlation

NF1 is caused by inactivating germline mutations in the neurofibromin gene on chromosome subband 17q11.2.5–7. Neurofibromatosis is an autosomic dominant disease. Penetrance, or the likelihood that the individual carrying the mutation will manifest the disease, is complete. However, NF1 is highly variable in its expression (ie, the severity of disease varies among affected individuals within the same family and from one family to another). No obvious genotype-phenotype correlation has been demonstrated in patients. To date only two correlations of clinical significance have been reported: the exon 17 del AAT associated with absent of neurofibromas (described above) [3] and the NF1 microdeletion phenotype [2]. About 1 to 5 percent of patients with NF1 have large deletions that encompass more than 700 kb of DNA and include the entire *NF1* gene. Such patients have a higher incidence of intellectual disability (mental retardation), developmental delay, some dysmorphic facial features, earlier appearance of cutaneous neurofibromas, and connective tissue abnormalities [51]. These patients have a paucity of cutaneous, subcutaneous, and superficial plexiform neurofibromas, that may relate to the fact that this mutation only deletes a single amino acid, unlike most other mutations that truncate or prevent Nf1 formation.

There is an increase role for NF1 genotyping in clinical practice. Family studies have suggested that the variation in expression seen in the majority of NF1 families is caused by the influence of modifying genes [52].

Moreover, the extreme clinical variability of NF1 suggests that random events are also important in determining the phenotype of affected individuals. Evidence in support of this interpretation is provided by the occurrence of acquired "second hit" mutations or loss of heterozygosity (LOH) at the *NF1* locus in some instances in the following tumors and other lesions characteristic of NF1:

- Neurofibromas [53]
- Malignant peripheral nerve sheath tumors [54]
- Pheochromocytomas [55]
- Astrocytomas [56]
- Gastrointestinal stromal [57]
- Myeloid malignancies from individuals with NF1 [58]
- Malignant melanomas [59]
- Mandibular giant cell granulomas [60]
- Glomus tumors [61]
- Tissue associated with tibial pseudarthrosis [62].

It seems likely that the clinical variability of NF1 results from a combination of genetic, non-genetic, and stochastic factors. Such complexity and the diversity of constitutional *NF1* mutations that occur in this disease will continue to make genotype-phenotype correlation difficult.

NF1 gene

To better understand the highly variable phenotype of NF1 and the complexity of this disorder is important investigate the structure of *NF1* gene and the pleomorphic function of the protein neurofibromin.

The NF1 gene (*NF1*; NM_000267.2) spans >350 kb of genomic DNA on chromosome subband 17q11.2.5–7 and comprises 57 constitutive exons and four alternatively spliced exons (9a, 10aII, 23a and 48a), encoding an 11- to 13-kb ubiquitous mRNA transcript [10, 11].

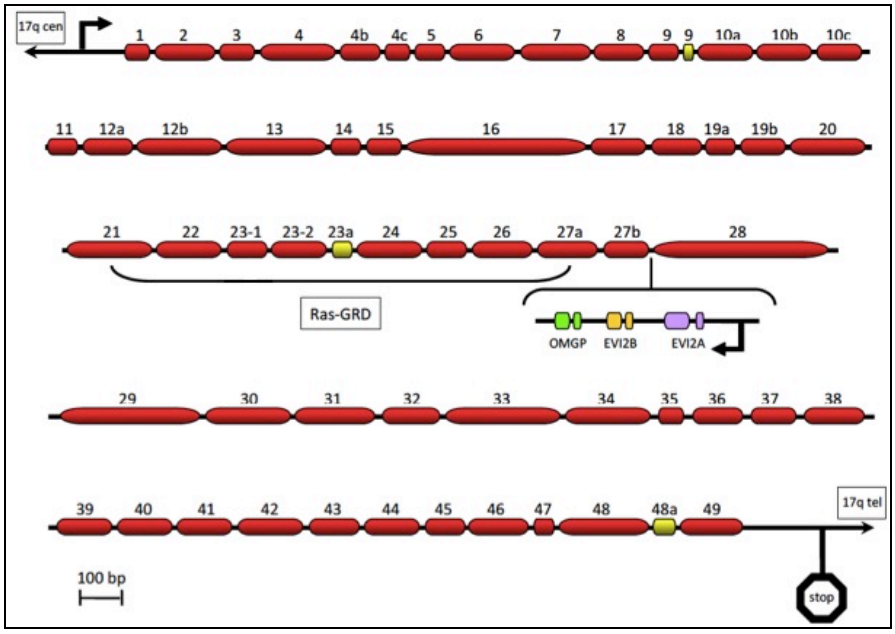

Figure 4. *NF1 gene structure.*

NF1 promoter region

To date, no pathogenic mutations have yet been reported in the promoter region [63, 64]. The NF1 gene promoter is located within a CpG-island-containing genomic region that exhibit a high degree of

sequence conservation with NF1 genes found in many other organism [65]. Hypermethylation of the NF1 promoter regions doesn't appear to be a common mechanism that inactivates the normal allele in any NF1-related tumors [66, 67].

3'Untraslated region (3' UTR)

The 3'UTR region is long 3.5 kb and exhibits a high level of sequence conservation, indicating its possible functional importance for regulating mRNA stability an for controlling translational efficiency of the gene.

Alternative splicing

The *NF1* gene has principally 4 alternative splicing (9a, 10a-2, 23a and 48a) that have been found to be differently expressed in various tissues in normal individuals.

The most common NF1 transcript is known as type II and it is conserved in several species[68]. Type II transcript includes the alternatively spliced 23a exon, that results in a 63 bp in-frame insertion in the GAP-related domain (GRD) of neurofibromin[69]. The resulting protein, with an additional 21 amino acids, exhibits a significantly reduced GAP activity but demonstrates an increased affinity for Ras in comparison to type I neurofibromin. Equivalent levels of type I and II *NF1* transcripts are ubiquitously expressed in normal tissue. Alternative spliced exon 9a, that inserts an additional 30 bp, is highly expressed in the central nervous system and it seems has a crucial role during embryonic development[70]. Instead Exon 48a is abundantly expressed in muscle and cause a 54bp in-frame insertion [71]. Controversial is the recognizing alternative splicing 10a-2, an

adding 45bp to the transcript, that was reported to be expressed in all human primary and tumor cells examinated at much lower level than type I transcript[72].

NF1 pseudogenes

NF1 gene displays seven partial pseudogenes sequences located on different human chromosomes (2q12-q13, 12q11, 14p11-q11, 15q11.2, 18p11.2, 21p11-q11 and 22p11-q11) [73], with at least two separate regions of sequence homology on both chromosome 15 and 22 [74]. Many of these pseudogenic sequence display significant homology (>90%), however they all have various inactivating nucleotide substitutions, insertions, or deletions. Pseudogenes result from multiple independent partial duplications of locus gene followed by sequential inter-chromosomal transposition events [75]. Pseudogenes sequence are not able to encode functional protein but they might act as potential reservoir of *NF1* mutations, and increase the *NF1* mutation rate by interchromosomal gene conversion events [76].

Genes within intron 27b

The *NF1* gene has to two large (>60 kb) introns: 1 and 27b. The intron 27b contains three small unrelated genes, *EVI2A, EVI2B* and *OMG,* each of which has two exons. Each of these genes is transcribed in the reverse orientation to the NF1 gene. EVI2A encodes a protein of 232 amino acids that is expressed in the brain and bone marrow. The *OMG* gene coded for a 416 amino acids cell adhesion protein expressed in oligodendrocytes. The dysfunction of this protein is also involved in multiple sclerosis (MS). Recently in literature, case

of patient with an association between neurofibromatosis and MS were reported.

Modifying genes in NF1

A possible role for modifier genes in NF1 disease phenotype expression was first proposed following the observation that monozygotic twins shared certain NF1 features [52, 77, 78]. By contrast, more distant affected relatives often exhibited more variable clinical phenotypes [52]. However, no germline modifying genes have yet been identified. A number of alternative mechanism have also been proposed to explain the clinical variability in NF1, including modifying genes, allelic heterogeneity, a mutation in the second allele, a somatic mosaicism and deletion of contiguous genes. The influence of environmental and stochastic factors had also been proposed to explain the marked inter-individual clinical phenotype variation [79].

NF1 Protein

The most common *NF1* transcript coded for neurofibromin, a cytosolic 2818-amino-acid polypeptide [80, 81]. Neurofibromin is a negative regulator of Ras guanosine triphosphate (GTP)ase proteins. Studies in vertebrates, showed that NF1 is ubiquitous express from the onset of organogenesis to mid-stage embrionyc development, while in adult organism it is expressed predominantly in neuronal cells of the brain, as neurons Schwann cells, astrocytes, oligodendrocytes and leukocytes [82, 83]. Homozygous deletion of *Nf1* gene is lethal in mice [84] and leads to size defects in fruit fly *Drosophila Melanogaster* [85], suggesting roles in organismal development. The subcellular localization of neurofibromin is cell type dependent: associated with the plasma membrane [86], the endoplasmic reticulum [87] and also colocalized with mitochondria [88].

Various biochemical approaches were used to identify other interaction partners other than Ras. These include tubulin [89], kinesin-1 [90], protein kinase A (PKA) [91], and C [92], syndecan [93], caveolin [94] and the amyloid precursor protein [95]. While regions of neurofibromin involved in those interactions have been reported, the biological significance of the respective protein-protein interactions remains unclear. Up to date NF1 protein was characterize by four main domain: Cystein and Serine Rich Domain (CSRD), Gap Region Domain (GRD), Leucine Zip Domain (LZD) and the C-terminal Domain that contain inside the Nuclear Localization Site (NLS) and Tyrosine Kinase Recognition sites. These regions were involved for protein interaction.

Main Protein Interactions

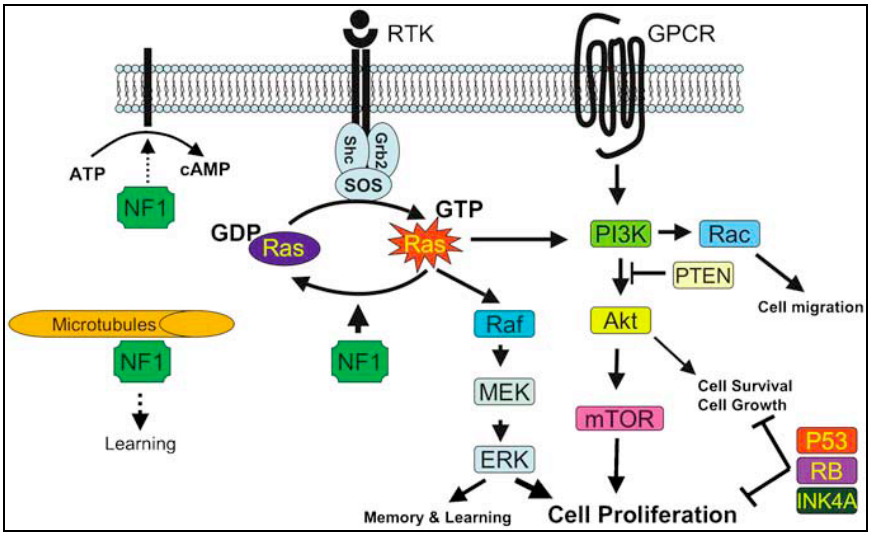

Figure 5. *Modified from "Tumor microenviromenment and neurofibromatosis type I: the GAPS"* [96].

Intracellular cyclic Adedosine Mono Phosphate (cAMP): Studies in Drosophila reveal a possible role of neurofibromin in a RAS-indipendentway. In particular, in Schwann cells the lack of NF1 could induce the amount of cAMP and inhibiting GTPase protein involved in cellular adhesion and synaptogenesis [97]. Other studies as referred that inhibition of cAMP/GTPase pathway of Rho-RAC and aberrant organization of cytoskeleton were at the origin of neuro-cognitive problems in NF1 patients [98]. The CSRD domain contains a number of potential cyclic adenosine monophosphate (cAMP)-dependent protein kinase A binding sites, indicating a possible role in cAMP signaling [91].

Protein Kinases: biochemical analysis reveal six possible serine-threonine sites for phosphorylation by PKA (more accreditable one) and one by PKC. The role of phosphorylation of neurofibromin by PKA was not well understood [99].

Tubulin interaction. It was shown that neurofibromin and tubulin interact and could compose a complex with low GTPase activity. This interaction may contribute to Ras regulation [89].

RAS/MAPK pathway

The RAS/mitogen-activated protein kinase (MAPK) pathway plays a vital role in development. *RAS* gene constitutes a multigene family that includes HRAS, NRAH and KRAS. RAS proteins are small guanosine nucleotide-bound GTPases that function as critical signaling hub within the cell. They are activated through growth factors binding to receptor tyrosine kinases (RTKs), G-protein-coupled receptors, cytokine receptors, and extracellular matrix receptors. Receptors activations trigger the recruitment of a complex containing the adapter protein growth factor receptor bound protein 2 (Grb2) and the Ras guanine nucleotide exchange factor Sos to the site of RTK activation. Here, Ras is catalyzed to switch to its GTP-bound state. This active form of Ras then binds and activates the kinase Raf and phosphatidylinositol 3-kinase (PI3K), which then sets off a kinase cascade, culminating the activation MAPK and PI3K pathways. Some of these signals are then transmitted to the nucleus, regulating the expression of genes controlling cell proliferation, cell death, differentiation and migration (Figure 5) [96]. For these implications, it is established that constitutively active mutations of Ras are frequent and

associated with multiple human cancers as a result of permanent stimulation of the Raf–MAPK and/or PI3K signaling cascades that lead to uncontrolled cell proliferation and escape of apoptosis [100]. Neurofibromin acts as a negative regulator of Ras signaling through the conversion of the active GTP-bound form of Ras in the cell to the inactive GDP-bound form of the protein. The NF1 protein is a member of a large family of evolutionary conserved proteins, the mammalian GTPase activating protein-related proteins (GAP-related proteins)[101] In particular its Gap Region domain (GRD), involved exons 20-27a, directly interacts with RAS and accelerates the conversion of the active, GTP-bound Ras into its inactive GDP bound form [11, 102]. For this role, NF1 is considered as tumor suppressor gene. In accordance with Knudson's two-hit hypothesis, inheriting one germline mutation of *NF1* gene is not sufficient to cause cancer development. A second hit in the wild-type *NF1* allele occurring somatically produces a tumor. In fact, the development of malignant cancers in Nf1 individuals requires further acquisition of additional genetic aberrations, whether it is inactivation of PTEN, TP53, CDKN2A or amplification of platelet-derived growth factor receptor or epidermal growth factor receptor [103].

Scope of the thesis

Neurofibromatosis type 1 (NF1) is a human autosomal dominant disorders that affects approximately 1 in 3,500 individuals worldwide. The most common features of NF1 are pigmentary abnormalities, such as cafe´-au-lait macules, skinfold freckling, Lisch nodules and cutaneous and plexiform neurofibromas (PNs). NF1 is the most common cancer predisposing syndrome affecting the nervous system. Glioma is the most common central nervous system neoplasia in NF1 patients: 15-20% NF1 children develop low grade optic gliomas. PNs occur in 30% of NF1 patients in peripheral nervous system. Patients with PNs have a 20-fold higher risk of developing malignant peripheral nerve sheath tumors (MPNSTs) than other NF1 patients. Mutation detection in the *NF1* gene is complex, due to the large size of the gene (>350 kb), the presence of pseudogenes, the lack of hot spots, and the great variety of possible mutations. Hence, the clinical and molecular diagnosis of *NF1* may be challenging and its fine-tuning is desirable.

Given the challenge of molecular diagnosis, our fist aim was review the ten years experience in *NF1* diagnostic area of our laboratory. More than 452 unrelated patients were referred to our Institute between 2003 and August 2013 with clinical suspicion of neurofibromatosis type 1. According to NIH diagnostic criteria (NIH, 1988) 297/452 unrelated patients exhibited typical NF1 features. *NF1* mutation analysis with DNA-based protocol permitted us to find

mutations in 208 of 297 of clinically diagnosed patients (detection rate: 70%).

The second aim was the set up of a new integrative DNA/RNA protocol to improve the sensibility of our detection potency, decrease the report timing and answer to the 400% increase of testing requests to our laboratory in the last 3 years. To this porpoise we focus on the development of a new RNA-sequencing approach that could highlight new mutations and correct classified (splicing mutations were under estimate by only DNA approach). Moreover, this new approach halves the testing timing.

Moreover, we describe a peculiar case family with a case of heterozygous compound. We describe a rigorous method to investigate the possible pathogenicity of unknown variant. It may be useful in patients with no fulfilling clinical criteria.

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Chapter 2

A ten year of Italian experience in Neurofibromatosis type 1: 102 novel mutations

INTRODUCTION

This report would describe our Italian diagnostic activity in the field of Neurofibromatosis 1 after 10 years of experience. In the last 10 years, we set up an efficient DNA-multistep protocol: Multiplex Ligation-Dependent Probe Amplification Analysis (MLPA) analysis, Denaturing high-performance liquid chromatography (DHPLC) and DNA sequencing (see M&M for more details). New technical approaches and expertise permitted us to improve our starting detection rate from 54% (2003-2008) to 70% (2008-2013). In 2008, we introduced MLPA analysis in our screening protocol. More than 452 unrelated patients were referred to our Institute between May 2003 and August 2013 with clinical suspicion of neurofibromatosis type 1. According to NIH diagnostic criteria (NIH, 1988) 297/452 unrelated patients fulfilling NF1 features. Mutation detection in the *NF1* gene is complex, due to the large size of the gene (>350 kb), the presence of pseudogenes, the lack of hot spots and the great variety of

possible mutations. Moreover, signs are age-dependent and present high variability in penetrance and expressivity even between affected members of a family. Hence, the clinical and molecular diagnosis of *NF1* may be challenging and its fine-tuning is desirable.

MATERIALS AND METHODS

Study population

Patients who referred to our Institute are from different Italian centers and the major part comes from North of Italy. 546 people were screened to our Institute between May 2003 and August 2013 for the genetic test of NF1. 452/546 were unrelated patients and 94 were familiar cases. In concern to NIH diagnostic criteria (NIH, 1988) 297/452 unrelated patients fulfilling NIH criteria. The median age is 21,2 years where 18,5% (55/297) had less than 5 years and we characterized 60% of sporadic case. In addition, we investigated 56 families (a cohort of 94 people) to define their condition in the familiar transmission (40/56 families) or to evaluate as pathogenic a sequence change of unknown clinical significance (16/56 families). All patients gave informed consent prior to genetic analysis.

DNA extraction

In brief, DNA was isolated from EDTA-blood samples using Gentra ®Puregene® Blood Core Kit B (Quiagen, Venlo, Netherlands).

Multiplex Ligation-Dependent Probe Amplification Analysis (MLPA)

MLPA was the first step of our screening protocol for *NF1* gene.

Patients' DNA were analyzed by MLPA with NF1 MLPA salsa P081 and P082 (MRC Holland, Amsterdam, The Netherland). P081/P082 salsa kit highlighted single- and multi-exon deletion/duplications inside NF1 gene. The P081 probemix-1 contains 38 MLPA probes and the P082 NF1 promix-2 contains 41 MLPA probes. The amplification products are between 130 nt and 463 nt that cover almost all 60 exons on *NF1* gene. P081/P082 positive patients for the entire NF1 deletion were screened also with MLPA P122 salsa kit to determine large rearrangement and microdeletion. Indeed, this kit reveled microdeletions that involved NF1's contiguous genes thank the presence of 25 probes for 17 different genes located at close distance to NF1. Results obtained by ABI Prism 3130 Genetic Analyzer (Life Tecnologies) were analyze with upgrade Coffaliser.Net Software (MRC Holland, Amsterdam, The Netherland). MLPA analyses were introduced from 2008.

Microarray-based comparative genomic hybridization (aCGH)

Abnormalities identified by MLPA were subsequently tested using array CGH Agilent (protocol CGH v.6.0). A custom-designed 8x60K array (https://earray.chem.agilent.com/earray/) was used to detect exons deletions and duplications in the *NF1* gene. The array had 28224 internal control probes were present along chromosome 17 (Design ID 026850) and 15888 in duplicate probes spanning the region of the *NF1*gene on chromosome 17q11.2. The vast number of probes permits oversampling of the sequence region. A custom-designed 6x44K array (https://earray.chem.agilent.com/earray/) was used for detecting gross deletions in 17q11.2. The array has

18446 internal control probes were present along chromosome 17 (Design ID 022022) and 12777 in duplicate probes spanning the region between 25,5 Mb and 27,8 Mb bases on chromosome 17q11.2. The average spacing between probes is 180 bases. CGH arrays were performed by the department of Human Pathology of Pavia.

DHPLC analysis

Genomic DNA of MLPA's negative patients were analyzed by Denaturing high-performance liquid chromatography (DHPLC). *NF1*s gene was amplified by PCR in 60 amplicons of around 400 bp. PCR product were analyzed on a DHPLC Wave Transgenomic 3500HT (Transgenomic®, Crewe, UK) equipped with a DNASep column (Transgenomic®, Crewe, UK). The oven temperatures for optimal heteroduplex separation were determined using the WAVEmaker software version 4.1.40 (Transgenomic®, Crewe, UK), which gives a computer- assisted determination of melting profile and analytical conditions for each fragment. Primers and annealing temperature were improved starting from Han et al., 2001[1]. In particular we redesigned 4 primers: 2 primer pairs (ex 1 and ex 7) were designed according to Upadhyaya et al 2004[2] and 2 primer pairs (23-2, and 30) werere newly designed to obtain improved PCR amplification (software "Primer Express" Applied Biosystems) (available on request). Those PCR products displaying a heterozygous pattern were sequenced by ABI Prism 3130 Genetic Analyzer (Life Tecnologies) to individuate mutation.

Sequencing approach

Sequencing analysis was then performed on DHPLC positive amplicons using ABI PRISM BigDye terminator sequencing kit v1.1 (Life Tecnologies) on an ABI Prism 3130 Genetic Analyzer (Life Tecnologies). Bidirectional DNA sequences were compared to healthy samples and referred to NM_000267.3 (NC_000017.10). The exon number was given according to the conventional rule used in the NF1 testing community and previous literatures[2,3]. Moreover parents or familiar study were usually screened for novel variation identified and not for all the NF1's gene (direct sequencing).

Predictions of possible impact at protein and mRNA level

The genetic screening based on genomic DNA (gDNA) need good predictive tools to study the possible effect on NF1 gene and protein for the genetic variation identified. Our analysis take the advantage of different databases and prediction sites starting from Mutation Taster (http://www.mutationtaster.org) that gave you a lot of information querying simultaneously different databases as NCBI SNP database (dbSNP), *1000 Genomes Project* (TGP), disease variants from dbSNP (ClinVar) and Human Genome Mutation Database (HGMD) [4]. In a second time, HGMD (Human Genome Mutation Database – Institute of Medical Genetics, Cardiff, Wales, UK; http://www.hgmd.cf.ac.uk/ac/index.php) and LOVD (Leiden Open Variation Database- http://www.LOVD.nl.NF1) NF1's mutation database were usually interrogate to define as novel the mutation identified. If the alteration is not known in literature, the prediction analysis continued investigate the impact of amino acid

substitution on the structure and function modifications (PolyPhen-2, https://genetics.bwh.harvard.edu./pph2/;)[5]. Otherwise the possible effect on mRNA (canonical and not canonical splicing mutation) were evaluated with Splice site Prediction by neural network (htpp://www.fruitfly.org/seq_tools/splice.html;)[6], the Human Splicing Finder (HSF; http:www.umd.be/HSF/;)[7] and ESE Finder tools(http://rulai.cshl.edu/cgibin/tools/ESE3/esefinder.cgi?process=home;)[8,9].

RESULTS

We identified the pathogenic NF1 mutation in 243/452 unrelated patients (Figure 1b) submitted for clinical genetic testing to Neurological Institute C. Besta. Analyzing our cohort of unrelated patients (452) we showed that 297/452 were fulfilling NIH criteria. Our DNA-based protocol permitted us to detect the pathogenic mutation in the 208/297 patients (detection rate 70%).
Moreover, 35 mutations were identified among the 155/452 that didn't fulfill the clinical criteria at the moment of the analysis request. Notable, 21/35 (60%) were children under 5 years and only 8 individuals were adults (median age 11,5 years).

On the other hand, 94 individuals were analyzed as familiar cases. Among the 56 families we studied 40 families to verify the familiar proband and the possible transmission inside the family component. Parents of 16 young children were screened to verify pathogenicity of missense alteration identify (Figure 1a).

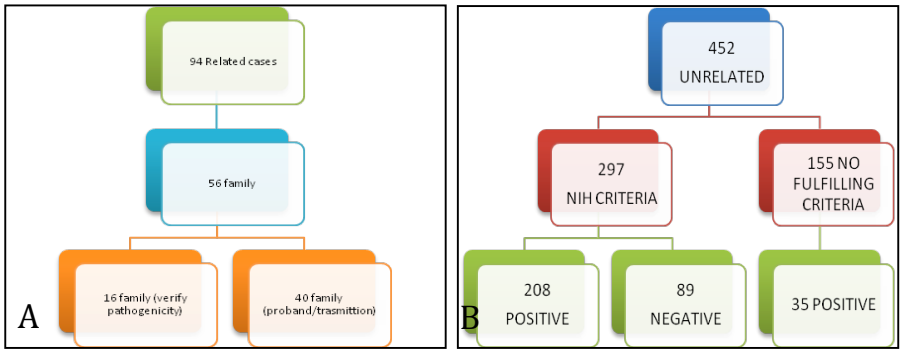

Figure 1. *Characterization flow chart of related (A) and unrelated (B) patients of our cohort.*

Among the 452 unrelated patients we could identify 187 different single mutations. 15/187 were microdeletion or atypical deletions/duplications of one or more exons. Instead 172/187 were small changes that spread among all *NF1* gene 1-49 exon (Figure 2 and Figure 3) [2,3]. Looking the position of the 172 small mutations, no real hot spot region where identified but some exons were more prone to accumulate mutations (Figure2). Amplicon 20, 21 and 29 had 7 while exon 28 Amplicon 20, 21 and 29 had 7 is the most hit exon in our cohort. 13 exons had between 4 to 6 alterations; 34 exons display 1 to 3 changes and last exon 12b,19a, 23a, 27b, 34, 35, 47, 48a, 49 weren't involved in changes (Figure 2).

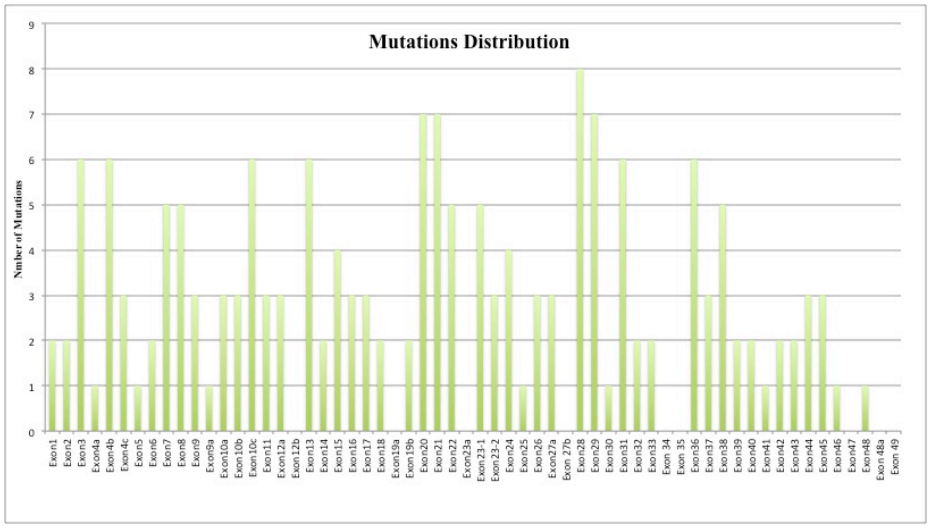

Figure 2. *Distributions of the 172 single small mutations identified for each exon.*

It was well known in literature that *NF1* gene has a high rate of variations, indeed, 163/187 (87%) single mutations were defined as "private" (only observe in one unrelated patient/family). Only 24/187 single mutations (13%) were observed more than once in us cohort of 452 unrelated people. The most frequent mutation has been identified in 7 unrelated patients: c.1466A>G, p.Tyr489* (exon 10b) [10]. In other five unrelated patients we detected a nonsense variation (c.4084 C>T; p.R1262*) in the exon 23-2 reported by Upadhyaya and colleagues in 2007[2].

Furthermore we characterized all our 187 mutations and we discovered that 102 (54,5%) were defined as novel. We defined as "novel" variations that were not present in the main database used in the field: HGMD and LOVD. Novel mutations have been deposited in the LOVD database

(https://grenada.lumc.nl/LOVD2/mendelian_genes/home.php?selectdb=NF1) and they were described by HGSV recommendations. Missense mutations were checked in 100 healthy people to avoid single nucleotide polimorphisms (SNPs).

In Table 1 we summarized the 102 novel mutations identified in our cohort of patients (A- Small changes; B- gross alterations). Instead, in table 2 we reported "known" mutations and their frequency (A- small changes; B- gross alterations).

Patient	Position	Mutation	Protein Prediction	Clinical Features	Class/Effect
NF1_239	E1	c.21_22delGG,	p.Glu8Metfs*29	CALs, AF, LN, NF	splicing
NF1_317-570	E2	c.99 A>G	p.?	CALs, AF, LN	splicing
NF1_428	E2	c.185dupT	p.Leu62Phefs*5	CALs, LN, U'bos,macrocephaly, behavior problems	Ins
NF1_177-483	E3	c.240_243del TCTC	p.Gln83*	CALs, NF, AF, LD, hypotyrodism, short stature	del
NF1_314 - 315	E3	c. 247delCinsGAGA	p.Gln83delinsGluLys	CALs, NF,LN,	del/ins
NF1_499	E3	c. 252delG	p.Ile85Leufs*18	CALs, NF,LN, familiar NF1	del
NF1_136	IVS3	c.288+1delG	p.?	CALs, NF,LN, AF, heart attack	splicing
NF1_354	E4a	c. 479 G>T	p.Arg160Met	CALs,AF, NF, LN	splicing
NF1_330	E4b	c. 529_530insGA	p.Ile177Argfs*2	AF, NF, LD, White matter hyperhintensity	ins
NF1_82	E4c	c.647_649delTGG	p.Leu216_Lys217delinsGln	CALs,AF,plexiform NF,LN	del
NF1_384	E4c	c.652_653delAAinsG	p.Lys218Glyfs*7	CALs,AF, NF,LN, familiar NF1	del/ins
NF1_228	E6	c. 755 T>A	p.Leu252*	CALs, NF	nosense
NF1_333	E7	c.953_956delAAAG	p.Glu318Valfs*57	CALs, sclerosing cholangitis	del

Patient	Position	Mutation	Protein Prediction	Clinical Features	Class/Effect
NF1_355	IVS7	c. 1062+113 A>G	p.Asn355Valfs*12	3 CALs, optic pathway glioma dx,	splicing
NF1_173	IVS7	c. 1063-2A>C	p.?	CALs,AF, LD, abdominal plexiform	splicing
NF1_219	E8	c. 1105C>T	p.Gln369*	NF,intertriginous freckling axillary, ciscoliosis,short stature	nosense
NF1_195	E8	c. 1140delT	p.Val381Phefs*6	CALs, AF, LN	del
NF1_111	E8	c. 1174 C>T	p.Gln392*	CALs, AF	nosense
NF1_385	E8	c. 1180dupT	p.Lys395*	CALs,Ubos, lentigo	ins
NF1_341-609	E9	c.1260dupT	p.Ser421Phefs*8	CALs, AF, OG (?)	ins
NF1_269	E10b	c.1520delT	p.Leu507Cysfs*19	CALs,AF	del
NF1_144	E10c	c.1552_1553delAC	p.Thr518Profs*39	NA	del
NF1_193	E10c	c. 1555delC	p.Gln519Lysfs*7	CALs, LD, epilepsy, LN	del
NF1_254	E10c	c. 1603 C>T	p.Gln535*	CALs, AF, OG, psycomotor retardation	nosense
NF1_434-640	E10c	c. 1634 C>A	p.Ala545Glu	CALs,AF,pulmonary stenosis mild, Brain MR gliosis, familiar NF1	missense
NF1_135	E11	c. 1713_1716delGGGA	p.Trp571*	CALs, NF,AF	del

Patient	Position	Mutation	Protein Prediction	Clinical Features	Class/Effect
NF1_372	IVS11	c. 1722-2 A>T	p.?	CALs, bony dysplasia,mild psycomotor retardation	splicing
NF1_208	E12a	c. 1722 C>G	p.Ser574Arg	CALs, AF, brainstem glioma	missense
NF1_220	E13	c. 2019delC	p.Cys673*	CALs, NF, Brain MRI: hamartomas and/or gliomas	del
NF1_450	E13	c. 2067_2070delGGCC	p.Ala690Cysfs*57	CALs, familiar NF1	del
NF1_349	E13	c.2084_2085delTG	p.Trp696Glufs*3	CALs,AF, NF, LN,LD.	del
NF1_488-489	E13	c. 2205 T>G	p.Tyr735*	CALs,AF, familiar NF1	nosense
NF1_171	E14	c. 2325 G>C	p.Glu775Asp	CALs, NF,AF, LN,LD.	splicing
NF1_558	E15	c. 2356 del C	p.Gln786Lysfs*5	CALs,AF, scoliosis	del
NF1_416	IVS15	c. 2410 -18 C>G	p.Gln803fs*23	CALs, NF,AF, familiar NF1	splicing
NF1_441	E16	c. 2846 del G	p.Gly949Aspfs*5	CALs	del
NF1_487	E17	c.2870delA	p.Asn957Ilefs*5	CALs, AF	del
NF1_244	E19b	c. 3278 T>A	p.Val1093Glu	CALs, 1 intertriginous freckling,LD	missense
NF1_407	E20	c. 3335 delA	p.Asn1112Thrfs*3	CALs,AF, NF.	del
NF1_8-399/120	E20	c. 3337 del C	p.Leu1113Phefs*2	familiar NF1	del

Patient	Position	Mutation	Protein Prediction	Clinical Features	Class/Effect
NF1_459	E20	c. 3384_3390delTGGCAGG	p.Gly1129Asnfs*11	CALs,AF, NF, LN, bony dysplasia	del
NF1_3	E20	c. 3456_3459delACTC	p.Leu1153Metfs*4	NF, familiar NF1	del
NF1_89	E21	c.3523 A>G	p.Thr1175Ala	CALs,AF, bony dysplasia, brainstem hamartoma, familiar NF1	missense
NF1_394	E21	c.3586 C>G	p.Leu1196Val	CALs, AF.	missense
NF1_117	E21	c.3644 T>G	p.Met1215Arg	CALs, OG	missense
NF1_216	E22	c.3844delA	p.Ser1282Valfs*3	CALs, AF,LN, OG	del
NF1_104	IVS 23-1	c. 3974+1G>A	p.?	CALs, NF,AF, LN,LD , familiar NF1	splicing
NF1_225	IVS 23-1	c. 3974 +2 T>G	p.?	CALs,NF, scoliosis	splicing
NF1_328-376	E23-2	c.3995delT	p.Glu1333Argfs*10	CALs, macrocephaly, hypertelorism, familiar NF1	del
NF1_99	E24	c. 4118_4119delGC	p.Ser1373Thrfs*29	CALs,AF,NF, LN,LD	del
NF1_339	E24	c. 4236_4237delTAinsGATT	p.Arg1413Ilefs*7	CALs,AF, LN.	del/ins
NF1_408	IVS24	c. 4269+2 T>G	p. ?	CALs, LD, brainstem glioma, lentigo	splicing
NF1_153	E25	c.4301delT	p.Phe1434Serfs*14	NF, optic pathway gliomas, scoliosis	del

Patient	Position	Mutation	Protein Prediction	Clinical Features	Class/Effect
NF1_70	E26	c.4381A>G	p.Ile1461Val	CALs, hypertension ,mild mental retardation, macrocephaly, hydrocefalus	missense
NF1_30	E27a	c. 4606dupA	p.Thr1536Asnfs*7	CALs, NF,AF, LN, LD, macrocephaly, mental retardation	ins
NF1_156 F	E28	c. 4830_4844del	p.Lys1611_Ala1615del	CALs,AF,bony displasia	del
NF1_424	E28	c. 4859 T>C	p.Ile1620Thr	CALs, familiar NF1	missense
NF1_305	E28	c. 4867G>C	p.Asp1623His	CALs, AF	missense
NF1_403	E28	c. 5047 A>T	p.Lys1683*	CALs.	nosense
NF1_32	E28	c. 5154_5158dupATTC	p.His1720Ilefs*17	CALs, NF,AF e LN, familiar NF1 ,hypertension	ins
NF1_41	E28	c. 5162 A>G	p.Asn1721Ser	CALs,AF, NF	missense
NF1_506	E29	c. 5472insT	p.Arg1825Serfs*16	CALs, NF, ,AF, LN, OG	ins
NF1_49	E31	c. 5890 G>T	p.Glu1964*	CALs, NF,AF, LN .	nonsense
NF1_360	E31	c. 5927 G>A	p.Trp1976*	CALs,AF, NF, LN, familiar NF1	nosense
NF1_319	E31	c. 5943G>T	p.Gln1981His	CALs,AF, NF, plexiformNf	missense
NF1_206	IVS31	c. 5944-1 G>T	p. ?	CALs, NF,AF,LN, plexiformNf	splicing

Patient	Position	Mutation	Protein Prediction	Clinical Features	Class/Effect
NF1_10	E32	c.5944-?_6084+?dup341	p.?	LD	dup
NF1_197	E32	c. 6084G>C	p.Lys2028Asn	CALs, NF,AF, LN	missense
NF1_112	E33	c. 6347 C>G	p.Ser2116*	CALs, pulmunary stenosis, small intersettal rhabdomyomas	nosense
NF1_6	E36	c.6642-?_6756+?dup140	p.?	Cals, NF, OG, familiar NF1	dup
NF1_7	E36	c. 6688delG	p.Val2230Serfs*14	NF, familiar NF1	del
NF1_393	E36	c. 6692_6693delTT	p. Phe2231Trpfs*4	CALs, familiar NF1	del
NF1_2	E38	c. 6907C>T	p.Gln2303*	NA	nosense
NF1_23	E38	c. 6938 G>A	p.Gly2313Asp	CALs, NF	missense
NF1_29	IVS38	c. 6999 +1 G>C	p.?	CALs, NF, AF	splicing
NF1_340-413	IVS38	c. 6999 +1 G>T	p.?	familiar NF1	splicing
NF1_460	IVS38	c. 7000-1 G>C	p.?	CALs,AF, LN, Ubos	splicing
NF1_231-313	IVS39	c. 7126 +2 T>G	p.?	CALs, NF, familiar NF1	splicing
NF1_90	E40	c. 7190_7191delCT	p.Leu2398Glyfs*2	CALs, AF, LD	del
NF1_553-427-600	E40	c. 7240 A>T	p.Ser2414Cys	CALs, LD	missense

Patient	Position	Mutation	Protein Prediction	Clinical Features	Class/Effect
NF1_79	E41	c. 7309dupA	p.Arg2437Lysfs*8	CALs, NF	ins
NF1_78	E42	c. 7425_7426insTCTC	p.Pro2476Serfs*6	NF, severe scoliosis	ins
NF1_463	E42	c. 7519 delC	p.Gln2507Asnfs*20	CALs, AF	del
NF1_404-622	E43	c. 7580dupA	p.Ser2528Ilefs*7	CALs, familiar NF1	ins
NF1_201	E43	c. 7619 C>G	p.Ser2540*	CALs, ,LN, AF	nosense
NF1_322	E44	c. 7793 T>G	p.Leu2598Arg	CALs,AF, NF, scoliosis	missense
NF1_210-211	IVS44	c. 7806+1 G>A	p.?	CALs, AF, LN, familiar NF1	splicing?
NF1_169	IVS45	c. 7907 +1 G>T	p.?	CALs, NF	splicing?
NF1_552-427	E46	c. 7994 A>G	p.Gln2665Arg	NF	missense
NF1_202	E48	c. 8111delC	p.Pro2704Glnfs*14	CALs, familiar NF1,AF, chiasmatic OG	del

Table 1 A) *New small mutations: Café au lait spot (CALs), neurofibromas (NF), axillary freckling (AF), optic gliomas (OG), Lisch nodules (LN), Learning disabilities (LD), Not available (NA), exon (E), intron (IVS)*

Patient	Deleted	Type of deletions	Clinical Features
NF1_31	ATAD5 - ex1 NF1	Atypic (1,8 Mb)	CALs, NF, LN, MR, DFF
NF1_226	CRLF3, RAB11, RP4	Atypic (0,871Mb)	Cals, AF, NF
NF1_505	BLMH-NF1	Atypic (1,1 Mb)	CALs, AF
NF1_582	RNF135-NF1	Atypic (0,4Mb)	CALs, AF, LN, scoliosis

Patient	Deleted	Type of deletions	Clinical Features
NF1_95-96-373-374	27a	single exon (1,215kb)	CALs, AF, NF
NF1_267	10c-23a	multiple exon (35 kb)	CALs
NF1_451-494	10b-14	multiple exon(13kb)	CALs,AF, NF, LN
NF1_377	23-2/ 23a	multiple exon(4 kb)	CALs,AF, LN, OG
NF1_468	1+ promoter	single exon (4 kb)	CALs,AF, NF, LN
NF1_521	4c-8	multiple exon(19.6 kb)	CALs,AF, NF, LD

Patient	Duplication	Type of duplication	Clinical Features
NF1_602	22/23-1	multiple exon (0,3 kb)	CLS, NF, AF
NF1_295	10b/23a	multiple exon (45 kb)	CLS

Table 1. B) *New atypical microdeletion and deletions/duplication of one or more exons. Café-au-lait (CALs), neurofibromas (NF), axillary freckling (AF), Optic glioma (OG), Lisch nodules (LN), Learning disabilities (LD), mental retardation (MR), dysmorphic facial features (DFF), Not available (NA), Exon(E), Intron (IVS).*

Patient	Frequency	Position	Mutation	Protein Prediction	Reference
NF1_446	1	E1	c.27 G>A	p.Trp9*	LOVD
NF1_34	1	E3	c.236 T>G	p.Leu79*	LOVD
NF1_469	1	E3	c.247 C>T	p.Gln83*	[11]
NF1_121-323	2	E4b	c.484 C>T	p.Gln162*	[12]
NF1_22-(232-233)-323	3	E4b	c.495_498delTGTT	p.Thr166fs*11	[11]
NF1_412	1	E4b	c. 499_502del TGTT	p.Thr167Glnfs*9	13
NF1_66 -74 - 125	3	E4b	c.574 C>T	p.Arg192*	[13]
NF1_28	1	IVS 4b	c. 586+5 G>A	p.Leu161fs*2	[14]
NF1_461	1	E4c	c.649delG	p.Glu217Lysfs*8	[10]
NF1_310	1	E5	c.663 G>A	p.Trp221*	LOVD
NF1_(106/251)	1	E6	c. 809 A>C	p.Gln270Pro	LOVD
NF1_87	1	E7	c.910 C>T	p.Arg304*	[15]
NF1_(179-501)	1	E7	c. 1019_1020delCT	p.Ser340Cysfs*12	[16]
NF1_312	1	E7	c. 1021_1022 delGT	p.Val341Hisfs*11	[17]
NF1_316	1	E9	c.1246 C>T	p.Arg416*	[11]

Patient	Frequency	Position	Mutation	Protein Prediction	Reference
NF1_462	1	IVS9	c. 1260 +1 G>A	p.Ser421fs	[18]
NF1_447	1	E9a	c.1260 + 1604 A>G	p.Ser421Leufs*4	[19]
NF1_241-281-356-425	4	E10a	c. 1318 C>T	p.Arg440*	[20]
NF1_280-392	2	E10a	c. 1381 C>T	p.Arg461*	[21]
NF1_168	1	E10a	c. 1392 +1G>T	p.Ser421_Pro464del	[22]
NF1_259	1	E10b	c.1399 insA	p.Thr467Asnfs*3	[23]
NF1_46-48-157-199-229-245- 279	7	E10b	c.1466 A>G	p.Tyr489*	[11]
NF1_100-218	2	E10c	c.1541_1542delAG	p.Gln514fs*21	[24]
NF1_203	1	E10c	c.1542delG	p.Lys514fs*10	[25]
NF1_370-432	2	IVS11	c. 1721+3 A>G	p.Ala548fs	26
NF1_162-(318-422)-423	3	E12a	c. 1756_1759delACTA	p.Thr586fs*17	[27]
NF1_522	1	IVS12a	c. 1846+1 G>A	p.?	[28]
NF1_304	1	E13	c. 2033dup	p.Ile679Aspfs*21	[20]
NF1_421	1	E13	c. 2041 C>T	p.Arg681*	[14]

Patient	Frequency	Position	Mutation	Protein Prediction	Reference
NF1_212	1	E14	c. 2291 T>C	p.Leu763Pro	[21]
NF1_272	1	IVS15	c. 2409+1 G>A	p.?	[29]
NF1_344	1	IVS15	c. 2409+1 G>T	p.?	[29]
NF1_290-291	2	E16	c. 2541 T>C	p.Leu847Pro	[30]
NF1_471	1	IVS16	c.2990+1 G>C	p.Leu952fs	22
NF1_435	1	IVS17	c. 2991-2 A>G	Tyr998_Arg1038del	[11]
NF1_448	1	IVS17	c. 2991-1 G>A	p.?	[31]
NF1_(242-464-465)	1	E18	c. 3047_3048delGT	p.Cys1016fs*3	[29]
NF1_298	1	IVS18	c. 3198 -2 A>G	p.Asp1067fs*20	[32]
NF1_311	1	E19b	c. 3311 T>G	p.Leu1104Arg	LOVD
NF1_85	1	E20	c. 3449 C>G	p.Ser1150*	LOVD
NF1_3	1	E20	c. 3456 del ACTC	p.Leu1153fs*3	LOVD
NF1_559	1	IVS20	c. 3496 +2 T>C	p.?	[33]
NF1_438	1	E21	c.3520 C>T	p.Gln1174*	[30]
NF1_84	1	E21	c.3525_3256del AA	p.Arg1176fs	[21]

Patient	Frequency	Position	Mutation	Protein Prediction	Reference
NF1_54	1	E21	c.3610 T>C	p.Arg1204Trp	[14]
NF1_45-73	1+f	IVS21	c.3708+1G>C	p.?	LOVD
NF1_454	1	E22	c 3721 C>T	p.Arg1241*	[21]
NF1_509	1	E22	c.3739_3742delTGTT	p.Phe1247Ilefs*18	[21]
NF1_(200-452)-302-398	3	E22	c. 3826 C>T	p.Arg1276*	[20]
NF1_167	1	E22	c. 3847delA	p.Ile1284*	[17]
NF1_19	1	E23-1	c. 3916 C>T	p.Arg1306*	[27]
NF1_429	1	E23-1	c. 3941 G>A	p.Trp1314*	[16]
NF1_383	1	IVS23-1	c.3975 -2 A>G	p.Arg1325fs	[16]
NF1_110	1	E23-2	c.4054 del A	p.Ser1352fs*3	LOVD
NF1_134-152-204-303-308	5	E23-2	c.4084 C>T	p.Arg1362*	[16]
NF1_375	1	E24	c. 4172 G>C	p.Arg1391Thr	LOVD
NF1_327	1	E26	c.4440 delC	p.Asp1480Glufs*73	LOVD
NF1_283	1	IVS26	c. 4515-1 G>A	p.?	[3]

Patient	Frequency	Position	Mutation	Protein Prediction	Reference
NF1_528 -170	2	E27a	c. 4537 C>T	p.Arg1513*	[34]
NF1_273	1	E27a	c. 4630delA	p.Thr1544Profs*9	[35]
NF1_439	1	E28	c. 5172 G>A	p.Lys1724Lys	[21]
NF1_55	1	IVS28	c. 5206-2 A>G	p.?	[36]
NF1_391	1	E29	c. 5234 C>G	p.Ser1745*	[32]
NF1_222-271-276-306	4	E29	c. 5242 C>T	p.Arg1748*	[37]
NF1_379	1	E29	c. 5353 C>T	p.Gln1785*	[21]
NF1_369	1	E29	c. 5425 C>T	p.Arg1809Cys	[38]
NF1_213	1	E29	c. 5546 G>A	p.Gly1737fs	[23]
NF1_502	1	IVS29	c. 5546+5 G>C	p?	[29]
NF1_433	1	E30	c. 5624 C>G,	p.Ser1875*	LOVD
NF1_(299-409)	1	E31	c. 5839 C>T	p.Arg1947*	[39]
NF1_252-284-294	3	E31	c. 5896 C>T	p.Gln1966*	[30]
NF1_531		IVS33	c. 6364+2 T>A	p.?	LOVD
NF1_133-253-430	3	E36	c. 6709 C>T	p.Arg2237*	[21]

Patient	Frequency	Position	Mutation	Protein Prediction	Reference
NF1_52	1	IVS36	c. 6756+1 G>A	p.?	[21]
NF1_426	1	IVS36	c. 6756+2 T>C	p.Phe2215fs	[10]
NF1_458-182-183	2	E37	c .6789_6792delTTAC	p.Tyr2264fs*4	[40]
NF1_(277-550)-147	2	E37	c. 6791insA	p.Tyr2264*	[41]
NF1_246-350	2	E37	c. 6792 C>A	p.Tyr2264*	[40]
NF1_257	1	E39	c. 7096_7101delAACTTT	p.Asn2366_Phe2367del	[42]
NF1_331	1	E44	c. 7720 delA	p.Val2575Phefs*28	[23]
NF1_33-65-77	3	E45	c. 7846 C>T	p.Arg2616*	[35]
NF1_293	1	IVS45	c. 7907+5 G>A	p.?	[17]

Table 2. A) *Small known mutations identified and frequency in our cohort.*

Patients	Microdeletion/duplication Known
NF1_12-57-98-264-275-307-371-510-607-621-641	Microdeletion Type I 1,4Mb
NF1_227-75	Microdeletion Type III 0,9 Mb
NF1_573	Duplication exon 2/3 (3 kb)

Table 2.B) *Microdeletion and duplication of exons described in literature.*

Among the 102 novel mutations 90 were small changes while 12 were aberrant deletions/duplications of one or more exons. The 90 small changes mutation spread along all *NF1* gene among exon 1 and 49. We couldn't characterize any hot spot region for novel mutations inside our cohort.

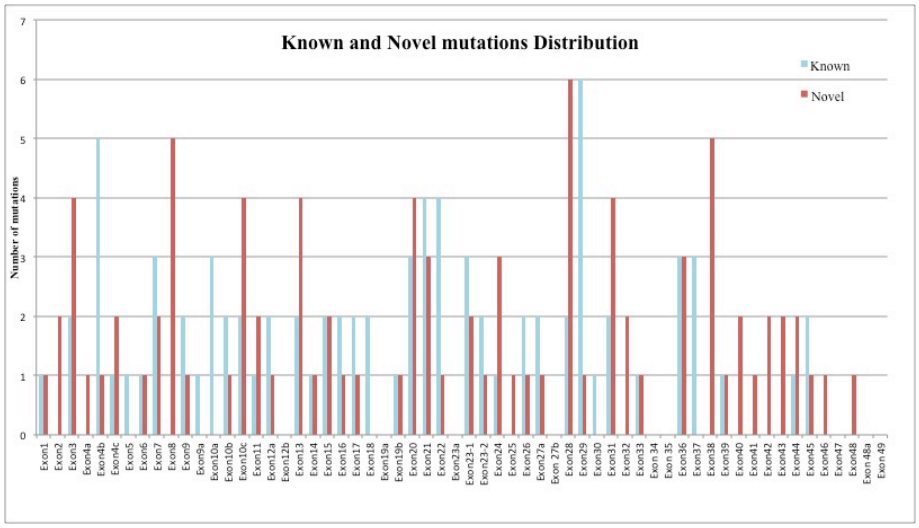

Figure 8. *Distributions of the 172 single small mutations divide in Known(82) and Novel mutations (90) for each exon.*

Few papers individuate region or domain of *NF1* involved in accumulation of mutations. We divided our small change variations (90/102 new mutations) in concern of NF1 protein domain trying to better characterize a possible link between mutation and NF1 molecular alterations. Up to date NF1 protein was characterize by four main domain: Cystein and Serine Rich Domain (CSRD), Gap Region Domain (GRD), Leucine Zip Domain (LZD) , C-terminal Domain (CTD) that contain inside the Nuclear Localization Site (NLS) and Tyrosine Kinase Recognition sites (TRS).

Main Protein Domain	Second Domain	Aminoacid Involved	N° of variation
CSRD		543-909	6
GRD		1168-1530	12
LZD		1543-1550	0
CTD		2260-2818	20
	NLS	2534-2550	2
	tyr recognition site		

Table 3. *Mutations in NF1 protein domain.*

We divided our variations inside these 4 domains (Table 3) and we observed a preference for accumulation of mutation in the GRD (12/90 – 13%) domain and the CTD (20/90 – 22%). No novel mutations damaged LZR or TRS sites, instead two alterations modified NLS domain. Only the 42% (40/90) of variations damage *NF1* domain while the other 58% hit exons that apparently, at the moment, had no biological importance. We also test a "tertile division" witch divide the *NF1* gene in three main regions the NF1

gene: the 5' tertile (exons 1-16), the middle region that contain the GRD domain (exons 17-30) and the 3' tertile (exons 31-49) [43]. Furthermore this division didn't reflect significant results (Table 4).

Tertile	Exons region	N° of variation	Percent
5'	1-16	36	40
Middle	17-30	26	29
3'	31-49	28	31

Table 4. *Mutations and tertile division of NF1 gene.*

As second step, we focus our attention on the kind of mutation distinguished in our cohort: the majority of the identified mutations consist of small changes (90/102 – 88%). The most prevalent mutations were small deletions and insertions (43% - 45/102) almost always causing frameshifts and premature stop codons, followed by splicing (21% - 21/102), nonsense (11% - 11/102) and missense (13% - 13/102) mutations (Figure 4). Microdeletion and deletions/insertions of one or more exon represent the 12% (12/102).

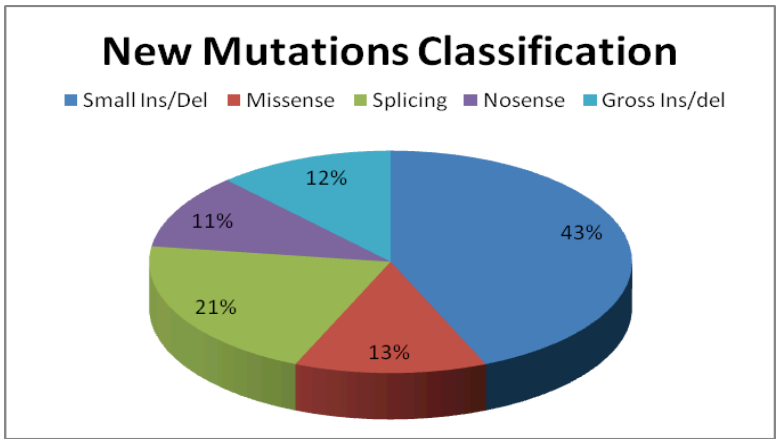

Figure 4. *The classification of 102 novel mutations*.

Small deletion and insertion

Small deletions were the most frequent alteration in *NF1* gene (43% - 45/102) almost always causing frameshifts and premature stop codons. Only 2/45 patients had in frame deletions (NF1_82; NF1_156F). Small deletions were characterized for deletion of 1 or 2 nucleotides at most (23/45). Few deletions involved more than 3 nucleotides. Two exceptions: NF1_459 lost 7 nucleotide in the exon 20 and NF1_156F lacked even 15 nucleotides of exon 28. Instead 10/12 patients showed insertion or duplication of 1 or 2 nucleotides. We found the duplication of part of exon 32 and exon 36 in two patients. Moreover it was possible also distinguished 3 more complex alterations as deletion and insertions of new nucleotides that caused frameshifts and truncated protein (NF1_314/315, 384, 339). See Table 1 for more details.

Single nucleotide change mutations: nonsense, missense and silent mutations

Truncated protein were, also, cause by nonsense mutations. In the novel mutations identified, 11 alterations (11%) caused stop codone triplets and produced truncated protein, data in line with the literature. Moreover, 13 mutations were classified as missense: we used PolyPhen software to predict if the amino acid variations were pathogenic in relation to how is conserved in evolution. We had a higher percent of missense variation than reported in literature (13 vs 7.4%)[22,49,50]. This different data could be cause by our DNA-approach protocol: RNA studies reveals that missense and silent mutations could alter the correct splicing if they hit consensus sites[10]. Evaluating nucleotide changing positions, 4/13 missense mutations should cause splicing mutation: NF1_208 and NF1_197 hit splice site consensus sequence; NF1_434/640 and NF1_322 where 8/10 nucleotides from exon-end, possible Exonic Splicing Enhancer sequences (ESE) that influence correct splicing form. RNA analyzes should evidence eventual splicing alterations. No new silent mutation where identified.

Splicing mutations: effect on mRNA

Two studies of Wimmer in 2007 and Pros in 2008 well characterized splicing alterations in *NF1* gene[10,44]. Moreover they defined 5 principal class on mRNA effect: I) exon skipping caused by mutation in 5' and 3' splice sites; II) inclusion of cryptic exon due to deep intronic mutations; III) new splice sites created by alterations within exons; IV) partial deletion of an exon or partial insertion of an intron induced by inactivating the canonical 5' and 3' Splice sites and

promoting the use of cryptic exonic or intronic splice sites; V) exon skipping caused to exonic mutation. Follow this classification we could individuate and further characterize our splicing mutation. Splicing mutation identified in our cohort predominantly hit consensus splice sites (type I and IV) in the donor site 65% (5'SS) 15/21 (Figure 5). 6/21 splicing mutation affect the first nucleotide of intron that is a well conserved "G" nucleotide. Exon skipping is usually associated with this alteration.

NF1_355 was characterized for a deep intronic alteration in the exon 7 (c. 1062+123 A>G). In *silico* program underlined that this mutation create a new donor site (type II). Specific primer revealed inclusion of cryptic exon in mRNA transcript and this induced a truncated protein at 366aa. Instead, NF1_416 (c.2410-18 C>G) caused the formation of a new aceptor site 18 nucleotides before the canonical one (type IV).

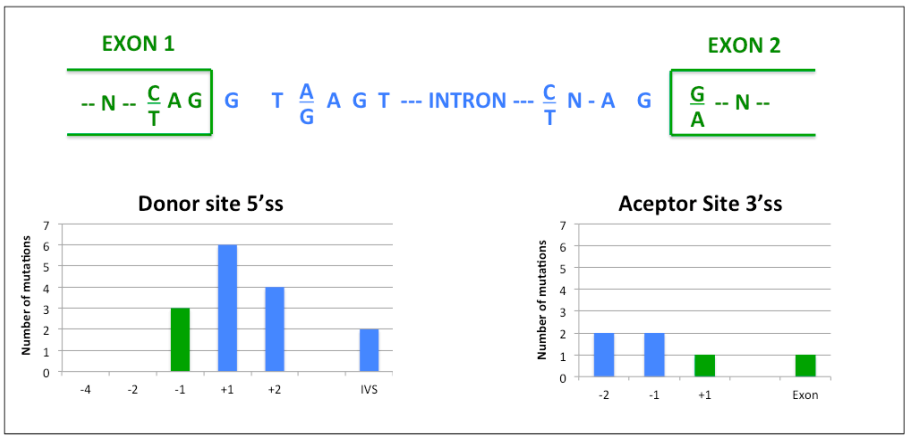

Figure 5. *Number of splicing mutations in donor and aceptor sites.*

Microdeletion and intragenic deletion/duplication of one or more exon (10%)

Twelve patients showed deletions or exon duplications that were reveled thanks MLPA analysis on DNA (Table 1b). This technique is very sensitive to detect restricted rearrangements that would have been missed by cDNA/DNA sequencing. Gross rearrangements were confirmed by CGH-array analysis.

In literature is reported that 5-10% of patients with neurofibromatosis type 1 have gross deletion. We distinguished 3/12 atypical microdeletions that involved the *NF1* gene and flaking regions at centromeric and telomeric region of chromosome 17q11.2. These deletions were extended from 0.8 to 1.8Mb and include different genes as SUZ12P, ATAD5, UTP6, SUZ12, NUFIP2, BLMH, LRRC37B (Table 1b). Moreover, we detected new intragenic (7/12) and intergenic (2/12) aberrant rearrangements. Intragenic deletion involved from 1 exon to over 35kb. Two distinct familiar cases showed the same internal deletion of exon 27a and the transmission to the offspring: NF1_373 transmitted it to his daughter (NF1_374), while NF1_95 transmitted to his son (and NF1_96). These 4 patients had a high clinical variability: from only CALs (NF1_373) to more severe complication as prostate cancer (NF1_95). Instead, 2 patients displayed duplication of more than one exon: NF1_602 from 22 to 23.1 exons (0.3kb) and NF1_295 from exon 10b to exon 23a (45kb).

In addition to atypical ones, we identified intergenic deletion: NF1_582 had a deletion of 0.4 Mb that extend from RNF135 to NF1 gene. Deletion could hit NF1 gene and contiguous genes at both side of chromosome 17 (telomeric and centromeric).

DISCUSSION

Here we report our Italian diagnostic experience in *NF1* gene in the last 10 years. We performed analysis in 452 unrelated individuals. Only 297 have typical NF1 features: CALs, axillary and/or inguinal freckling, cutaneous and plexiform neurofibromas (NFs), Lisch nodules of the iris, optic gliomas, specific bone lesions and increased risk of malignant tumors. Among the 297 index cases our DNA-based protocols revealed 208 positive patients (70% detection rate). However, 35 individuals with not fulfilling clinical criteria, at the moment of diagnosis, showed mutations. 24/35 were children of less than 8 years old (69%). In children, the clinical diagnosis can be more problematical because only the 50% of sporadic cases meet NIH criteria by the age of 1 year. Many features of NF1 increase in frequency with age and almost all do by the age of 8 years. However, among the 35 clinical negatives 8 were adult people with more than 32 years. In particular, we described the alterations for two patients with suspicion of NF1 and strange medical situations. NF1_10 has a duplication of 341 nucleotides in exon 32 while NF_355 has a splicing alteration (c.1062+113G>A) that induced the inclusion of a cryptic exon. NF1_10 patient at the clinic analysis shows a brain lesion that develop in astrocytoma in the time and hydrocephalus instead the second one manifested 3 CALs, an optic glioma and a mild scoliosis. Individuals with duplication of the entire NF1 locus and surrounding genomic region do not have the NF1 phenotype (which is caused by NF1 microdeletion or loss of function mutations) but may have intellectual disability and seizures[45,46]. The NF1 microduplication phenotype is variable, and some individuals with this genomic lesion

appear normal. Because several genes in addition to NF1 are involved in the microduplication, it is impossible to know whether the phenotypic abnormalities, when they occur, result from triplication of NF1 or of another gene(s) within the affected region[47]. Two uncertain clinical diagnoses that were characterized by genetic test. Potency of screening test is very important in an autosomic dominant disease, as Neurofibromatosis 1, that is predisposing to cancer. An early diagnosis in children and in atypical clinical signs could offer the better medical care at patients with multisystem problems.

We identified 187 distinct mutations. 24/187 mutations were described in more than 1 unrelated person while the others 164/187 were private. These alterations were spread along all *NF1* gene without any hot region. The long length of the gene (350kb), the presence of pseudogenes and the high rate of mutation increase the possibility of random hit. Few papers individuate region or domain of *NF1* involved in accumulation of mutations. Recently Bolcekova et al. proposed a correlation between clustering 5'tertile (1-16 exons) mutation and optic glioma in Slovak patients[48]. No more groups confirmed this association. We preferred to divide our variations in concern of NF1 domain trying to better characterize a possible link between mutation and NF1 molecular alteration and function. Up to date NF1 protein was characterize by four main domain: Cystein and Serine Rich Domain (CSRD), Gap Region Domain (GRD), Leucine Zip Domain (LZD) and the C-terminal Domain (CTD) that contain inside the Nuclear Localization Site (NLS) and Tyrosine Kinase Recognition sites (TRS). We divided our variations inside these 4 main domains (Table 3) and we observed a preference for

accumulation of mutation in the GRD (15/97 – 15%) domain and the CTD (19/97 – 19%). No novel mutations damaged LZR or TRS domain, instead two alterations modified NLS domain. Only the 42% damage *NF1* domain while the other 58% hit exons that apparently, at the moment, had no biological importance. Characterization of mutations' localization among NF1's domain could give information and predicted pathogenic effect in the biological functions of this multifaceted gene.

Two main genetic databases (HGMD and LOVD) were consulted for characterized our 187 mutations. Our laboratory identified 102 (54%) novel mutations that were not describe in literature before. We submitted them to LOVD database to share data with others groups. The median age of this sub-cohort was younger (20 years) than reported in literature: 53/102 (51,9%) were under 16 and 23/53 under even 5 years old (27/53 under 8 years). Female and male were present in the same range. Most of the novel mutations were small changes (88%) where small deletions/insertions predominated (43%).

Deletion/insertion of few nucleotides (1 to 15) almost always causing frameshift and premature stop codone. NF1_6 displayed duplication of 124 nucleotides in the exon 15 while NF1_10 even 341 coding nucleotides in 32 exon. No significant phenotype correlation was seen beside these two patients. Moreover NF_10 showed a better phenotype in comparison of NF1_6. Premature stop were caused also by the 11 nonsense mutations identified. Stop codone hit exon in indiscriminant way and produced truncated neurofibromin protein with different length from 252 (NF1_228) to 2540 amino acids

(NF1_201) against the 2818 amino acid of the wild-type form. Impairment in protein structure could be induced also by splicing alterations. We recognized a preferential alteration in the quite conserved nucleotides of consensus splice site. Splicing alteration in donor (5') and aceptor (3') sites usually cause exon skipping. Donor (5') site were compromised by 13/21 (62%) of splicing mutations predict by bioinformatics tools. To note we found two alteration in deep intron sequence: NF1_355 had mutation in intron 7 (c.1062+113A>G) that cause insertion of a cryptic exon and a truncated protein (p.Asn355Valfs*12); NF1_416 (c.2410-18C>G) create a new acceptor site and introduced 17 nucleotides in the exon that cause, also this time, a frameshift of triplette lecture and a stop codone after 23 aminoacids (p.Gln803fs*23). These mutations were confirmed by RNA analysis of patients. Splicing mutations probably were underestimated in the number due the DNA-approach that our laboratory had used. We had a higher percent of missense variation than reported in literature (13 vs 7.4%)[49,50]. This different data could be cause by our DNA-approach protocol: RNA studies reveals that new missense and new silent mutations could alter the correct splicing if they hit consensus sites[10]. Evaluating nucleotide changing positions, four missense mutations should cause splicing mutation: NF1_208 and NF1_197 hit splice site consensus sequence; NF1_434/640 and NF1_322 where 8/10 nucleotides from exon-end, possible Exonic Splicing Enhancer sequences (ESE) that influence correct splicing form. In our laboratory we collect and storage sample of DNA of patient and only recently RNA samples. Further studies on RNA will confirm eventual splicing effect of these missense mutations on NF1

transcripts that were underestimated in DNA-approach. Sabbagh et colleagues verify that about the 31,1% of missense mutations actually disturb NF1 transcript splicing[22]. In order to correct classified *NF1* mutations and their protein effect RNA-based protocol should be set up in the future. Nevertheless, we classified as missense mutations 18 nucleotide changes.

Up to date, microdeletions and atypical deletions/duplications were one of the few alterations with a phenotypic correlation. Their represent the 12% of the NF1 alterations that we identified: a higher percent than the 4% reported in literature[22, 49].

Type-1 deletion (1.4 Mb) is the most frequent microdeletion (70-80%)[51-53]. Less frequent were type II (10-20% - 1.2 Mb) and rare case of type III (1.4-4% - 1.0 Mb) were reported[54]. This three group were characterized by their breakpoint position and extension. Indeed, atypical *NF1* deletions (8-10%- >0.5 Mb) were non-recurrent and also heterogeneous deletions in terms of their size and breakpoint position. In our database we reported 11 patients with type I, 2 with type III and no type II microdeletions (Table 2b-Know mutation). Atypical microdeletions and deletions/duplication of single or multiple exons, performed by MLPA analysis, accounted 12 novel mutations identified in our Italian cohort. Among these 12 aberrant deletions/duplications 7 were validated by CGH-array performed by the department of Human Pathology of Pavia (Table 1 B).

CONCLUSIONS:

The data collected in these ten years of experience enable us to give an overview of the genetic and clinical aspects of NF1 molecular diagnosis. Our detection rate (70%) was lower than that reported in literature because it was principal based on DNA-approach. Negative index cases could be study at RNA level to search alteration and possible splicing effect of missed; by whole-exome sequencing approach, using next-generation sequencing, to search causal mutations in NF1 associated loci in the genome (as *SPRED 1* gene). However, the presence of unknown mutations in NF1 locus in these patients cannot be fully excluded even though mosaicism cases were underestimate in neurofibromatosisis 1.

The high mutational rate and the variable clinical penetrance, even though in the same family, require a complete characterization of the new NF1 alterations that were discovered in different populations. Only if Neurofibromatois community adds up information we could understand more about this multi-systemic pathology and its molecular basis.

These observations also points out the need for a more comprehensive *NF1* molecular diagnostic strategy. Nowadays, we design a new integrative DNA/RNA approach to answer to the high request number of NF1 test per year and to improve our detection rate. RNA approach will help us to individuate aberrant rearrangements and splicing mutations that were missed with DNA analyses. Recently we used this novel integrated protocol in a cohort of 33 blood samples and we identified 30/33 mutations (detection rate: 91%). In the future we expect to reach the 90%-95%, as reported in literature.

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Chapter 3

Integrated genetic studies of Neurofibromatosis type 1.

INTRODUCTION

During 2003-2013 *NF1* mutation analysis of genomic DNA was performed in 452 patients using the multiplex ligation-dependent probe amplification (MLPA) to look for deletions or insertions located inside the *NF1* gene. Subjects who tested negative for MLPA were investigated using denaturing high pressure liquid chromatography (DHPLC) and sequencing DNA. This genetic protocol permitted us to find mutations in 208 of 297 of clinically diagnosed patients (detection rate: 70%). Although numerous *NF1* mutations have been identified, detecting mutations in *NF1* has proved challenging, due to the large size of the gene (>350 kb), the presence of pseudogenes, the lack of hot spots, and the great variety of possible mutations. Moreover, the *NF1* gene is subject to one of highest mutation rates known for human genes (1×10^{-4}), and in 50% of all NF1 patients the case is classified as sporadic.

Hence, the molecular diagnosis of *NF1* may be challenging and its fine-tuning is desirable. The second aim of my PhD project was to

improve our detection rate by developed a sensitive, integrated genetic protocol using MLPA and RNA-based sequencing.

MATERIALS and METHODS:

DNA Extraction and sequencing (see Materials and Methods of Chapter 2).

Multiplex Ligation-Dependent Probe Amplification Analysis (MLPA) (see Materials and Methods of Chapter 2).

RNA Extraction and Retrotranscription

Collection of RNA samples was started since 2011. A full *NF1*'s RNA based analysis was routinely introduced in our laboratory from September 2013. RNA samples were collected in Tempus Blood RNA Tube (Life Technologies) and extracted with Tempus™ Spin RNA Isolation Kit within 4 days. DNase treatment with Absolute RNA Wash Solution was performed for all samples during RNA extraction protocol. RNA samples (1ug) were reverse transcribed using 50 units of High-Capacity cDNA Reverse Transcription kit (Life Tecnologies) and 20 unit of RNAse inhibitor (Ambion®). *Beta2microglobulin's* amplification was used as quality control for retrotranscription reaction.

PCR amplification and Sequencing approach.

Then *NF1*'s cDNAs were full amplified with 23 overlapping PCR using Taq Gold Polymerase® (Applied Biosystems, Foster City, CA).

The fragments were designed (about 400 bp) in accordance with Valero et al. 2011 with few renovations as a new primer pairs for one fragment that cover exons 29 to exon 31 (Table 1) [1]. PCR products were purified using ExoSAP-IT® (USB Corporation USA) according to the manufacturer's protocol. PCR products were sequenced in both directions using ABI BigDye terminator sequencing kit v1.1 (Life Technologies) on an ABI Prism 3130 Genetic Analyzer (Life Tecnologies). cDNA variations were confirmed at DNA level and Exon 1 was usually sequence also on DNA for GC rich component.

RESULTS

Our laboratory is one of the few Italian's one that makes the genetic diagnosis of Neurofibromatosis and laboratory requests of the genetic test tripled in the last 3 years with almost 209 requests in the 2013. We developed a sensitive, integrated genetic protocol using MLPA and RNA-sequencing to improve our detection rate and decrease the timing of report.

MLPA assay was introduced and well established in our diagnostic protocol from August 2008. Hence we focus our work to set up and familiarized with the new approach of RNA-sequencing.

First of all we validated the sensitivity of our method in a small cohort of 22 patients that we previously identified the pathogenic alteration by DHPLC analysis (Table 1). RNA primers were able to detected small variations as deletions or insertions as well as missense and nonsense mutations. Moreover, this RNA-approach permitted us to appreciate and confirmed splicing variant of *NF1* transcript predicted by bioinformatics tools as for NF1_298, 311, 352, 355, 556, 624 and

741 (see Table 1).

The RNA-sequencing was able to detect 20/22 mutations with the designed primers. The only exception was NF1_272 (c.2409+1G>A). This DNA alteration induced a skipping of the exon 15, but our primers (F6 and F7) were positioned in a wrong way to underline this kind of splicing: new specific primers were drawn.

Patient	Position	DNA	RNA	Protein effect	Variant
NF1_183	E37	c.6789_6792delTTAC	r.6789_6792delUUAC	p.Tyr2264fs*4	frameshift
NF1_272	IVS 15	c.2409+1G>A	r.? (r.2326_2409del83)	p.(Ala776_Gln803del)	splicing
NF1_298	IVS19a	c.3198-2 A>G	r.3198_3199delAG	p.Asp1067fs*11	splicing
NF1_311	E19b	c.3311 T>G	r.3311_3314delUUAA	p.Leu1104Hysfs*6	splicing
NF1_352	E37	c6801 A>G	r.6757_6858del101	p.A2253_K2286del	splicing
NF1_355	E7	c.1062+113 A>G	r.1062ins113	p.Asn355Valfs*12	splicing
NF1_361	IVS6	negative	Negative	Negative	Negative
NF1_369	E29	c.5425C>T	r.5425C>U	p.Arg1809Cys	missense
NF1_439	E30	c.5681 T>G	r.5681U>G	p.Leu1894Arg	missense
NF1_442	E42	c.7506 C>T	r.7506C>U	p.Ser2502Ser	missense
NF1_506	E29	c.5471dupT	r.5471dupU	p.Arg1825Serfs*16	frameshift
NF1_517	E3	c.259_264delTTGGA TinsAA	r.259_264delUUGGA UinsAA	p.Leu87Lysfs*15	frameshift
NF1_552	E46	c.7994 A>G	r.7994A>G	p.Gln2665Arg	missense

Patient	Position	DNA	RNA	Protein effect	Variant
NF1_553	E40	c.7240 A>T	r.7240A>T	p.Ser2414Cys	missense
NF1_556	E22	c.3748C>A	r.3709_3749del40	p.Asp1237fs*13	splicing
NF1_558	E15	c.2356delC	r.2356delc	p.GLn786Lysfs*5	frameshift
NF1_571	E33	c.6311 T>C	r.6311U>C	p.Leu2104Pro	missense
NF1_624	E12a	c1845 G>A	r.[1642_1845del204, 1722_1845del124]	p.[Ala548_Lys615del, Ser574fs]	splicing
NF1_628	E41	c.7353 T>C	r.7353T>C	p.Pro2451Pro	missense
NF1_644	E4c	c.617_618 GA>AT	r.587_654del67	p.Glu196Glyfs*11	splicing
NF1_705	E23-1	c.3941 G>A	r.3941G>A	p.Trp1314*	nonsense
NF1_741	E7	c.945_946delGCinsAA	r.889_1062del173	p.Lys297_Lys354del	splicing

Table 1. *Mutation identified with DNA-protocol and confirmed by RNA-sequencing.*

RNA analysis allows the correct classification of alterations that affect splicing but mimic nonsense, missense or silent mutations. RNA sequencing confirmed silent mutations (without changing in the amino acid sequencing) in NF1_442 and NF1_628; instead, we identified a splicing alteration with exon skipping of exon 37, in NF1_352 patient, whom we initially classified as missense alteration using bioinformatics tools. Moreover we define as benign an intronic alteration in NF1_361 patient with DNA variant (c.889-21C>T) because it didn't cause any splicing alterations (Table 1).

After the validation of our cDNA fragment in know cases, we re-evaluated 6 patients, with fulfilling NIH criteria for neurofibromatosis, who were resulted negative at the previously DNA analysis and were recommended from our neurologist for a fulfilling NF1's clinic (Table 2). RNA screening highlighted alterations in 5/6 patients (83% detection rate). Two alterations were nonsense while other two mutations were splicing. In particular, NF1_367 has an alteration in exon 1 (c.58 C>T) that creates an early new 5' donor sites that induce the transduction of a small protein of only 38 amino acids. For NF1_540 no alterations were found in RNA transcripts. Furthermore, the r.3456_3459delACUC alteration produced a truncated protein of 1156aa and was identified in the RNA transcript of NF1_255 but any alterations on DNA in the exon 7 were recognized. The same result was replicate in the laboratory of dr.Zara at IRCSS Gaslini Hospital of Genova. DNA analysis confirmed the newly identified alterations.

Patient	RNA	PROTEIN effect	Variant	DNA (second analysis)
NF1_124	r.6494C>G	p.ser2165*	nonsense	c.6494C>G
NF1_270	r.4473G>A	p.Trp1491*	nonsense	c.4473G>A
NF1_352	r.6757_6858del101	p.A2253_K2286del	splicing	c.6801A>G
NF1_367	r.57_60del4	p.Glu19Aspfs*20	splicing	c.58C>T
NF1_255	r.3456_3459delACUC	p.Leu1152Tyrfs*4	frameshift	Negative
NF1_540	Negative	Negative	Negative	Negative

Table 2. *Re-evaluation of DNA negative patients with RNA-sequencing*

These results encouraged us to begin our diagnostic activity where DNA analysis (MLPA) and RNA sequencing were integrated in a sensitive multi-step protocol (Figure 1).

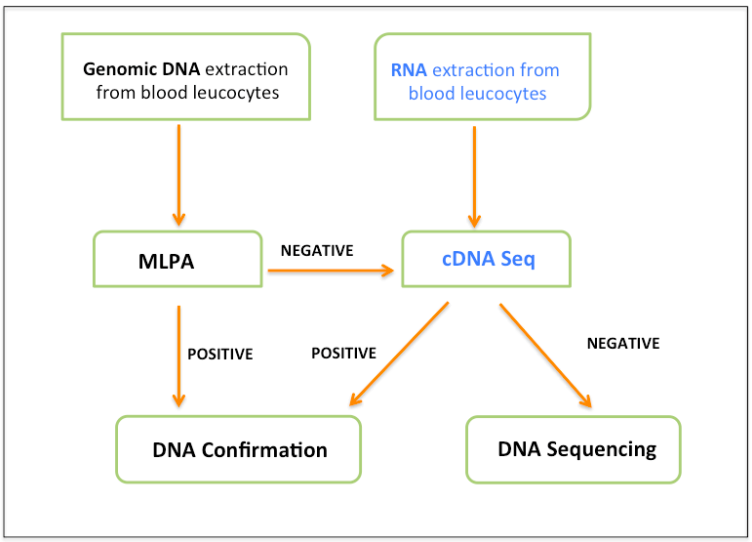

Figure 1. *DNA/RNA integrated protocol.*

As first step DNA patients were screened for deletion or duplications of one or more exons in addition to microdeletion of entire *NF1* gene and contiguous genes. During 2013 we evaluated 148 patients with MLPA assay and we founded alterations in 13 cases (8,8% of detection rate): 5/13 cases of microdeletion of type 1 (1,4Mb), 2/13 cases atypical deletions and 3/13 cases of partial exons deletion or duplications (Table 3). In details, NF1_582 has a deletion of 400kb that cover NF1 gene and RNF135 gene at the centromeric region; NF1_724 has a large deletion of 1,6 Mb deletion up to MYOD gene in teleomeric position than NF1. were also described (NF1_ 521, 650 and NF1_602). We detected also the deletion of exon 2 and 3 [2]in NF1_573 who had no fulfilling criteria at the moment of blood withdraw just for the early age of 8 moths.

In addition, MLPA assay was also able to detect small alterations as missense mutations (in the case of NF1_644 and NF1_712) and 1bp insertion (NF1_702) that were characterized in a second time by RNA-sequencing (3/13).

Patient	Deletions/Duplications	Reference
NF1_510	Microdeletion type 1	Dorschner et al. 2000; Jenne et al.2001; Lope´z-Correa et al. 2001
NF1_521	Deletion of 5 NF1's exons (19.6 kb: 4c-5-6-7-8)	Novel
NF1_573	Deletion exon 2 and 3 (3 kb)	(Upadhajaja 2006) [2]
NF1_582	Atypic deletion (400 kb) From RNF135 to NF1 gene	Novel
NF1_602	Duplication exon 22 e 23-1 (0.3 kb)	Novel
NF1_607	Microdeletion type 1	(Dorschner et al. 2000; Jenne et al.2001; Lope´z-Correa et al. 2001) [3-5]
NF1_621	Microdeletion type 1	(Dorschner et al. 2000; Jenne et al.2001; Lope´z-Correa et al. 2001) [3-5]
NF1_641	Microdeletion type 1	Dorschner et al. 2000; Jenne et al.2001; Lope´z-Correa et al. 2001
NF1_644	c.617_618GA>AT.	Novel
NF1_650	Deletion of 27 NF1's exons (60,6 kb fron 8 to 27a exon)	Novel
NF1_702	c.7422_7423insC	Novel
NF1_712	c.1466 A>G	(Osborn 1999)[6]
NF1_724	Atypic deletion (1,6 Mb): exon 4c NF1 to MYO1D gene	Novel
NF1_727	Microdeletion type 1	(Dorschner et al. 2000; Jenne et al.2001; Lope´z-Correa et al. 2001) [3-5]

Table 3. *Alterations identified by MLPA assay and references.*

As second step of our protocol, we evaluated through RNA-sequencing 25/135 negative cases of MLPA analysis with fulfilling NF1 criteria which RNA samples were available.

We were able to individuate 22/25 small pathogenic alterations (table 4). The kind of small alterations identified were well presented in this small cohort. The major part of them induces frameshift and truncated protein and just 2 alterations were missense that caused deleterious amino acid changing in protein sequence. Deletion and insertion of few nucleotides are the most frequent small changes individuated in NF1 patients.

Thanks to RNA-approach we appreciated 7 splicing mutations: 4 splicing mutations affected consensus splice site at the donor site (NF1_541, 695) and at the acceptor site (NF1_535, 740); the others 3 alterations hit nucleotide in the middle of the exon at +35/+44 nucleotide after the beginning of the exon (NF1_692, 707 and 754). All these splicing variants preferentially caused skipping of the entire exon. Only NF1_692 c.1885G>A creates a new donor site (5') at 40bp from the start of exon 12b. RNA-sequencing revealed deletion of the first 40bp and predicted a frameshift in protein sequencing that produced a truncated protein at 619aa.

Patient	Posision	DNA	RNA	Protein effect	Variant
507	E16	c.2493dupCA	r.2493dupCA	p.Asp832Glnfs*9	frameshift
527	E46	c.7993C>T	r.7993C>U	p.Gln2665*	nonsense
535	IVS17	c.2991-2A>C	r.2991_3113del123	p.Tyr998_Arg1038del	frameshift-in frame
536	E34	c.6477delC	r.6477delC	p.Ser2160Valfs*19	frameshift
541	IVS35	c.6641+1G>A	r.6579_6641del62	p.ala2194Ilefs*5	splicing
587	E19a	c.3163C>T	r.3163C>U	p.Gln1005*	nonsense
603	E37	c.6760delC	r.6760delC	p.Glu2255Argfs*4	frameshift
604	E37	c.6789_6792delTTAC	r.6789_6792delUUAC	p.Tyr2264Thrfs*4	frameshift
611	E22	c.3826C>T	r.3826C>U	p.Arg1276*	nonsense
614	E22	c.3826_3828delCGAinsTACT	r.3826_3828delCGAinsUACU	p.Arg1276Tyrfs*7	frameshift
623	E2	c.199dupA	r.199dupA	p.Asn67LysFs*9	frameshift
643		NEG	NEG	NEG	NEG
647		NEG	NEG	NEG	NEG
663	E13	c.2030dupC	r.2030dupC	p.Ile679Aspfs*21	frameshift
677	E24	c.4267A>G	r.4276A>G	p.Lys1423Glu	missense
691		NEG	NEG	NEG	NEG
692	E12b	c.1885G>A	r.1846_11886del40	p.Gln616Valfs*3	missense/splicing
695	E39	c.7126+3A>T	r.7000_7126del127	p.Ser2334Glyfs*21	splicing
696	E13	c.2041C>T	r.2041C>U	p.Arg681*	
700	700	c.3916C>T	r.3916C>U	p.Arg1306*	
707	E37	c.6801A>G	r.6757_6858del	p.Ala2253_Lys2286del	frameshift-in frame
720	E11	c.1658A>G	r.1658A>G	p.Hys553Arg	missense
740	IVS41	c.7395-2 A>G	r.7395_7552del158	p.Thr2466fs*7	splicing
754	E37	c.6792 C>A	r.6757_6858del102	p.Ala2253_Lys2286del	splicing in frame
755	E18	c.3037delA	r.3037delA	p.Thr1013Argfs*6	frameshift

Table 4. *Mutations identified by RNA-sequencing.*

DISCUSSION

Between 2003-2013 our laboratory used a DNA-based protocol to detect NF1 alterations. This protocol permitted us to reach a detection rate of only 70%. Moreover, in the last tree years, our laboratory tripled the requests of genetic testing for NF1 (209 only in 2013). These two triggers in addition with the advantage in technologies required establish of a new integrated and sensitive DNA/RNA protocol to decrease the timing of report and to increase detection of mutation. This method is more sensitive, especially in NF1 gene that has an high mutational spectrum and showing an high prevalence of splicing mutations not residing at the canonically conserved GT/AG splice sites. Indeed, mutations in the NF1 gene are spread over the entire coding region and include NF1 microdeletions, intragenic copy number changes, i.e. deletions/duplications involving one or several exons, frameshift, nonsense, splice, missense mutations and in frame deletions or duplications involving one to several codon[7-9].

First of all, we set up and improved a robust protocol of RNA-sequencing for screen all NF1 transcripts in a reliable and fast way in accordance with instrument present in the laboratory.

The validity of RNA-sequencing was confirmed in a cohort of 21 NF1 patients whose DNA alterations we characterized. The 23 PCR fragment designed allowed to detected small changes in RNA-sequencing and to reveal splicing variant caused by single nucleotide change in consensus or regulatory splice site [1]. Moreover, the potency of this approach was carried out well thanks the second study of 6 patients apparently negative at the DNA screening but clinically

fulfilling NF1 features. MLPA and DHPLC analysis screening were not able to detect the two nonsense and the two missense mutations with splicing effect reveled by RNA sequencing (Table 2). Probably, if all negative patients at MLPA/DHPLC analysis had been sequenced on DNA we would have been able to find more positive patients. However, the NF1 gene is length 350kb and composed by 57 constitutive exons and the entire sequencing on DNA is time and cost consuming in a small laboratory with diagnostic activity.

In the end, we applied to a small cohort the new integrated multi-step approach to analyze the entire NF1 coding region, using an RNA-based center assay, complemented with MLPA assay allowing to identify microdeletions and intragenic copy number alterations and to fully characterize the cDNA (coding DNA) alterations at the gDNA (genomic DNA) level. The new protocol consists in MLPA assay on DNA sample and sequencing of the negative samples though 23 PCR products fragments on the RNA transcript. We also plan to sequence all NF1 gene on DNA whom patients were result negative even if a strong clinical features of Neurofibromatosis.

In particular MLPA analysis was used as first screening step to find microdeletion and intragenic alterations. We choose this analysis, as first, for its accuracy, operating speed and for the lack of cytogenetic approach in our laboratory. 13/148 patients were positive to MLPA analysis with a detection rate of almost 9%. This percentage is higher than literature (4,5%) probably for the small cohort exanimated [7, 10] and maybe for the homogeneity of Italian individual in our group. In addition MLPA was able to detected 3/13 small changes in exon 4c, 10b and 42 and not only intragenic alterations. Microdeletions type 1

was the most frequent microdeletion (5/13) detected by P081/P082 mix of MLPA. Instead atypical deletion in NF1_724 and in NF1_582 were characterized by P122 mix. The combination of this two kind of assay permitted to reach the 9% of detection and individuate alterations even in NF1_573 who hasn't fulfilling clinic criteria at the moment of test analysis. All these remarks confirmed us the validity of this test as first analysis in our integrated protocol.

RNA-sequencing was performed on 25 MLPA's negative patients with fulfilling clinic criteria. This technique was able to detect the pathogenic alterations in 22 individuals with a final detection rate of 88%. Moreover the analysis and the sequencing of RNA allowed us to:

1) Correct classification of mutations that affect splicing but mimic nonsense, missense or silent mutations: incorrect classification for nonsense mutation, maybe effect on genotype/phenotype correlations. Without RNA-sequencing the mutation of NF1_707 should described as silent mutation while it produced a splicing variant that skip the entire exon 37 with a deletion in frame of 33 amino acids. Also NF1_754 were be erroneously classified as nonsense (p.Tyr2264*) while the splicing variant created has a deletion in frame as well as NF1_707 (Table 4). Moreover, incorrect classification could under-appreciate the formation of de novo splice sites witch are used despite the presence of intact wild-type splice sites and results in the loss of part of exon as for NF1_692, where a new 3' acceptor sites induced deletion of the first 40 nucleotides of exon 12b. Another class of exonic alterations leads to exonic skipping most likely by

disruption/creation of splicing regulatory elements such as exonic splicing enhancer (ESEs) or exonic splicing silencer (ESSs) [11, 12]. Although ESE/ESS prediction programs have proven to be useful for understanding the mechanism of mutation-induced exon skipping, experimental data from patients' samples remain pivotal to assess the effect on splicing of silent or conservative amino acid changes.

2) Avoidance of the detection of rare benign intronic variants with no significance (NF1_361 in Table1) and leading of benign exonic missense mutation with silent effect as NF1_442 and NF1_628 patients (Table 1).

3) Detection of deep intronic splice mutations: these mutations alter a single nucleotide often within very large introns, creating de novo 5' or 3' Intronic splice sites that are used in conjunction with an already available intronic "partner" cryptic splice site leading to inclusion of a cryptic exon [ref. 10 e 20]. In chapter 2 we describe a novel deep intronic splice site mutation in the intron 7. c.1062+113A>G in NF1_355 patient create a strong 5'ss that is used in conjunction with a cryptic strong 3'ss leading to inclusion of cryptic 113-bp exon containing a premature stop codon (Table 1).

4) Avoidance of amplification of non-processed pseudogenes.

CONCLUSION:

RNA-based mutation detection technique is a powerful, reliable and efficient means to identify mutations, especially in large and complex genes as NF1. Data obtain in the small group analysed, suggest us that integrated DNA/RNA-based protocols can improve our detection rate almost at 90% in patients suspected to have *NF1*. Up to date, in literature the most comprehensive *NF1* mutation protocol using RNA-based core assay supplemented with methods to identify NF1 microdeletions as well as smaller copy-number changes identifies mutations only in a 95% of non-founder NF1 patients fulfilling NIH criteria [7-9] On one hand, instability of RNA could under-appreciated the presence of some mutations and on the other the large intronic area which *NF1* gene is constitute could hide mutations in regulatory site that could influence the right transcription of NF1 as in NF1_255 who we are not able to identify genomic alteration . Integration of DNA and RNA approaches are needed for a sensitive genetic protocol. However, molecular diagnosis of *NF1* may be challenging: advantage in technologies with next-generations technique in the next future will give us a lot of new information about the complexity of this large and high mutational gene.

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Chapter 4

A family case report

INTRODUCTION

We have analyzed 452 unrelated patients affected by NF1 and 94 related individuals from 56 families since 2003. Most of them originated from Italy and 297/452 unrelated individuals fulfilled the international diagnostic criteria of NF1[1]. An extensive clinical and molecular examination was performed in all available first-degree relatives as well. Molecular analysis was carried out on a subset of patients and the causative mutation of the *NF1* gene was identified in 243/452 and 58/94 familiar cases. In this chapter, we present the clinical and molecular characterization of a child non-fulfilling the clinical phenotype of NF1 but presenting two novel missense mutations in exons 40 and 46. Further characterizations were extended to his family. We discuss the possible biological mechanism that could explain the *NF1* gene (trans) heterozygous compound.

MATERIALS and METHODS

Cell lines

Lymphoblast cell lines were obtained from peripheral lymphocytes of the patient and from healthy controls following Epstein-Barr Virus (EBV) infection and specifically selected with ciclosporin A (an immunosopressive agent) [2]. Cells were grown in vitro in Opti-MEM

(GIBCO, LifeTechnologies), 15% FCS I serum (Fetal Clone I, Thermoscientific), 2% sodium pyruvate (EuroClone-Milan, Italy), 2% penicillin-streptomycin (EuroClone-Milan, Italy), 1% non-essential aminoacids (EuroClone-Milan, Italy).

Western blot and antibodies

Protein samples were pelleted in RIPA lysis buffer with phosphatase and protease inhibitors, resolved using 10% SDS-PAGE and electroblotted onto PVC membranes. Membranes with transferred proteins were incubated with the primary antibody anti-NF1 (1:1000, sc-67, Santa Cruz, Germany), anti-ERk and anti-pERK (1:1000, Cell signaling), anti-AKT and pAKT (1:2000, Cell signaling) or anti-alphaTubulin (1:5000, Abcam, Cambridge, UK). The primary antibody incubation was followed by incubation with the secondary anti-mouse/rabbit antibody (1:10000). A chemiluminescence reaction using the ECL (enhanced chemiluminescence) Plus kit (Amersham, GE Healthcare, Buckinghamshire, UK) was detected using the G-Box detection system (Syngene).

Microarray analysis

Microarray analysis was performed on lymphocites of 3 NF1 patients, 3 individuals of the study family and 3 healthy controls.

Fragmented cRNAs were hybridized to the HTA 2.0 GeneChip (Affymetrix, Santa Clara, USA) following standard procedures. Data processing was mainly performed using Bioconductor 2.10 and R.15 [3]. The Robust Multichip Average [4] algorithm was applied to normalize using the quantile method, and normalized probeset intensities were calculated. A signal-based filtering was applied to the expression level

(>100) of each probeset for all of the different groups that were considered. Differentially expressed genes were identified using a fold change (FC) threshold of 1.2 for all sample comparisons. The functional annotation of genes that passed the FC and expression signal cut-offs was performed using the Gene Ontology (GO) Biological Process category and the hypergeometric test (hyperGTest function) [3] for gene over-representation.

In Silico prediction tools.

We took advantage of *in silico* prediction software to further characterize the two sequence variants of unknown clinical significance. GVGD combines the biophysical characteristics of amino acids and protein multiple sequence alignments to predict where missense substitutions in genes of interest fall in a spectrum from enriched deleterious to enriched neutral. Align-GVGD is an extension of the original Grantham differences to multiple sequence alignments and true simultaneous multiple comparisons [5].

GD>=65+Tan(10)x(GV^2.5)	Class C65	MOST likely to interfere
GD>=55+Tan(10)x(GV^2.0)	Class C55	
GD>=45+Tan(15)x(GV^1.7)	Class C45	
GD>=35+Tan(50)x(GV^1.1)	Class C35	
GD>=25+Tan(55)x(GV^0.95)	Class C25	
GD>=15+Tan(75)x(GV^0.6)	Class C15	
GD<15+Tan(75)x(GV^0.6)	Class C0	LESS likely to interfere

PolyPhen considers evolutionary conservation, physiochemical differences and the proximity of the substitution to predicted functional domains and/or structural features. Its scores can be classified as probably damaging (2.00), possibly damaging (1.50-1.99), potentially damaging (1.25-1.49), borderline (1.00-1.24), or benign (0.00-0.99) according to the classification proposed by Xi et al. [6, 7]. The user's own alignment cannot be used as an input for this tool.

SIFT is based on reference sequence alignments and produces scores which can be classified as intolerant (0.00-0.05), potentially intolerant (0.051-0.10), borderline (0.101-0.20), or tolerant (0.201-1.00) according to the classification proposed by Ng et al.[8] and Xi et al.[6]. NetPho predicts if the residue is not likely to be phosphorylated, either because the score is below the threshold or because the residue is not Ser, Thr, or Tyr. Moreover, the two missense mutations were checked on the dbSNP/EVS databases.

RESULTS

Clinical characterizations

A 2-years-old child (NF1_427) was referred to the infant neuropsychiatry of our hospital. His medical history revealed a neck dystonia few weeks after birth. He showed mild development disorder: he walked with aid at 15 months and independently at 18 months and displayed a delay in speech acquisition. In addition, the neurologic examination revealed macrocephaly, mild psychomotor retardation and four Café-au-lait spots on the back and the left leg. At

20 months he performed magnetic resonance imaging (MRI) and ophthalmological examination that failed to detect Lisch nodules or other pathological findings.

His father (NF1_552) was born prematurely at seven months. Neurological examination, brain and spinal MRI and ophtalmological evaluation were normal at 41 years of age. However he presented three neurofibromas at the level of the left leg, left groin and on the face.

His mother (NF1_553) had difficulties in reading at scholar age. At 43 years of age, she didn't shows any neurological or ophtamologic evidence of disease. However, she presented one CAL spot on the left gluteus. Moreover, family investigations revealed that her sister also had 2 CAL spots on the left and right gluteus and presented mild scoliosis and small iris nodules at the ophthalmological examination.

Mutational analysis

Mutational analysis for *NF1* gene, in NF1_427, revealed a double positivity for exon 40 and exon 46 by DHPLC investigation. The DNA sequencing detected two novel missense mutations: c.7240 A>T (exon 40) and c.7994A>G (exon 46). These mutations were also detected in RNA: missense mutations didn't affect any splice site but just caused the double amino acid changes p.Ser2414Cys and p.Gln2665Arg.

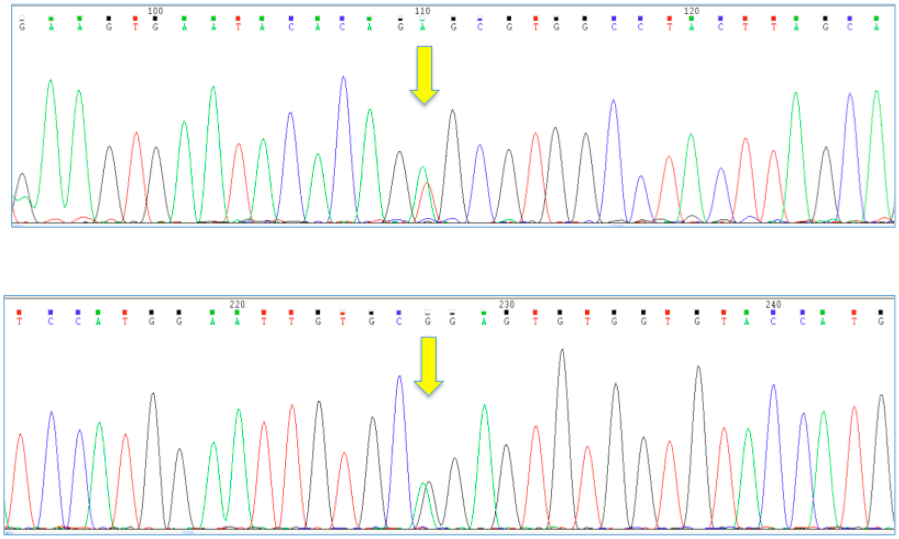

Figure 1. *Two missense mutations c.7240A>T (upper) and c.7994A>G (lower).*

Neurofibromatosis is an autosomic dominant mutation with 50% possibility to transmit the pathogenic alteration to the offspring. To evaluate the possible familiar transmission and the causative mutation we directly sequenced parent's DNA. Surprisingly, both were identified as positive: the mother had the c.7240 A>T transversion while the father showed transition c.7994 A>G. To exclude possible single nucleotide polymorphisms (SNP) we screened 140 healthy individuals (280 chromosomes) for both the mutations. We didn't find positive controls.

Molecular characterization

We further characterized the presence of two mutations, in *trans* position on NF1 gene, at the molecular level by gene expression profile. We obtain, after informed consensus, RNA from the NF1's

family members, 3 NF1's patients with nonsense mutation in the functional domain of neurofibromin (GRD-Ras domain) and 3 healthy individuals. All these 9 samples were hybridized on HTA 2.0 GeneChip (Affymetrix, Santa Clara, USA).

A first analysis showed that the child (NF1_427) has a unique profile that was dissonant from both healthy or disease person. Trying to identify a set of gene that characterized the NF1_427 condition we did a double comparison: we highlighted the genes with different expression (fold change>2) between the child and the NF1 patients but not in controls (Figure 2). We did the same analysis also in the opposite case (figure 3). We indentified 268 genes that were differently expressed from NF1_427 vs NF1's patients and 74 genes between NF1_427 and healthy controls. Gene Ontology class reveled genes involved in gene expression, intrinsic pathway for apoptosis, metabolism, generation of second messenger molecules and MAPK targets/Nuclear events mediated by MAP kinases moves in different ways in the two groups of controls.

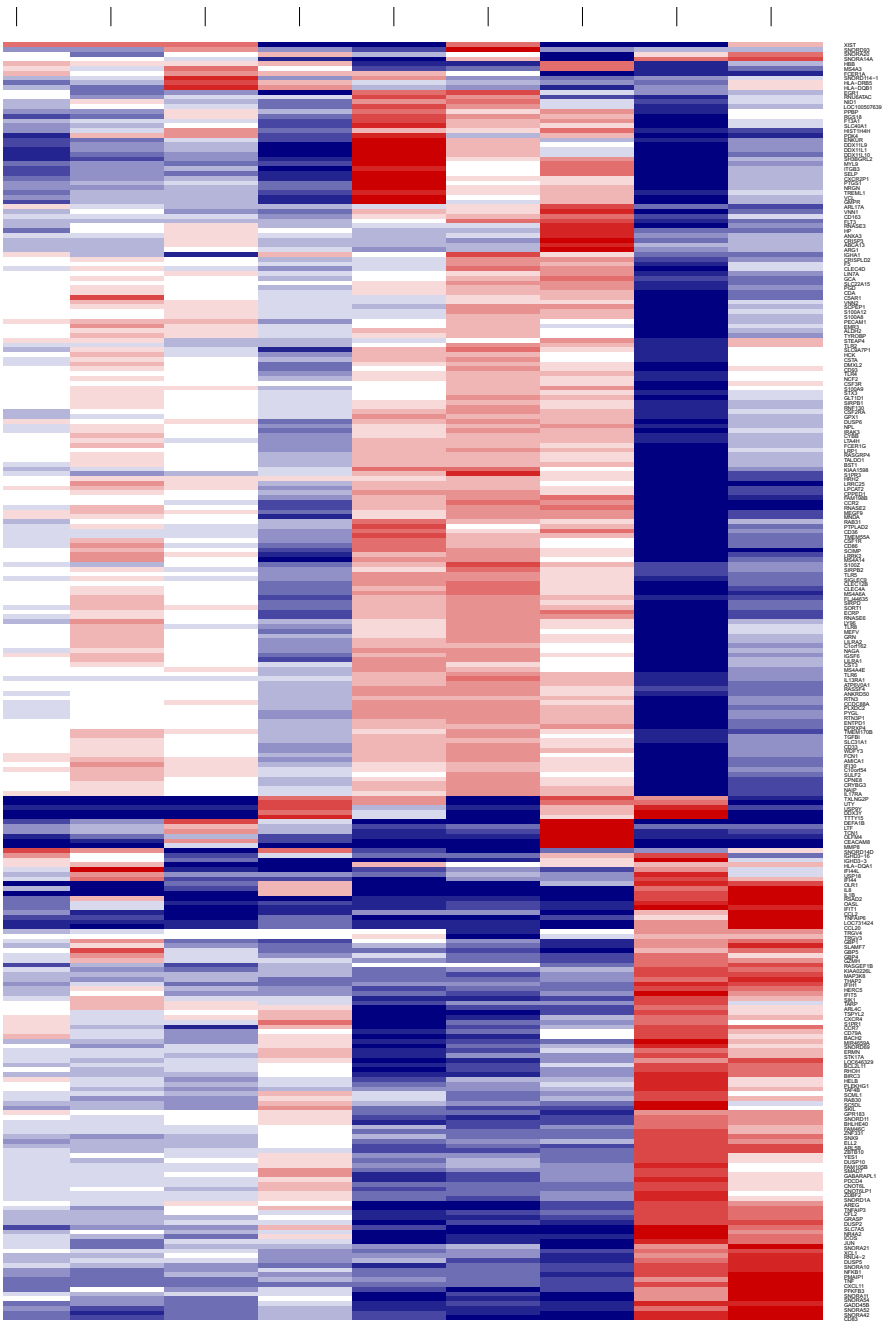

Figure 2. *Gene expression profile NF1_427 versus NF1's patients (FC>2). Healthy control -2<FC<2 From the left S002 (heathy), S003 (healthy), NF1_614 (NF1's patient), S001 (healthy), NF1_552 (father), NF1_303 (NF1's patient), NF1_744 (NF1's patient), NF1_427 (son) and NF1_553 (mother).*

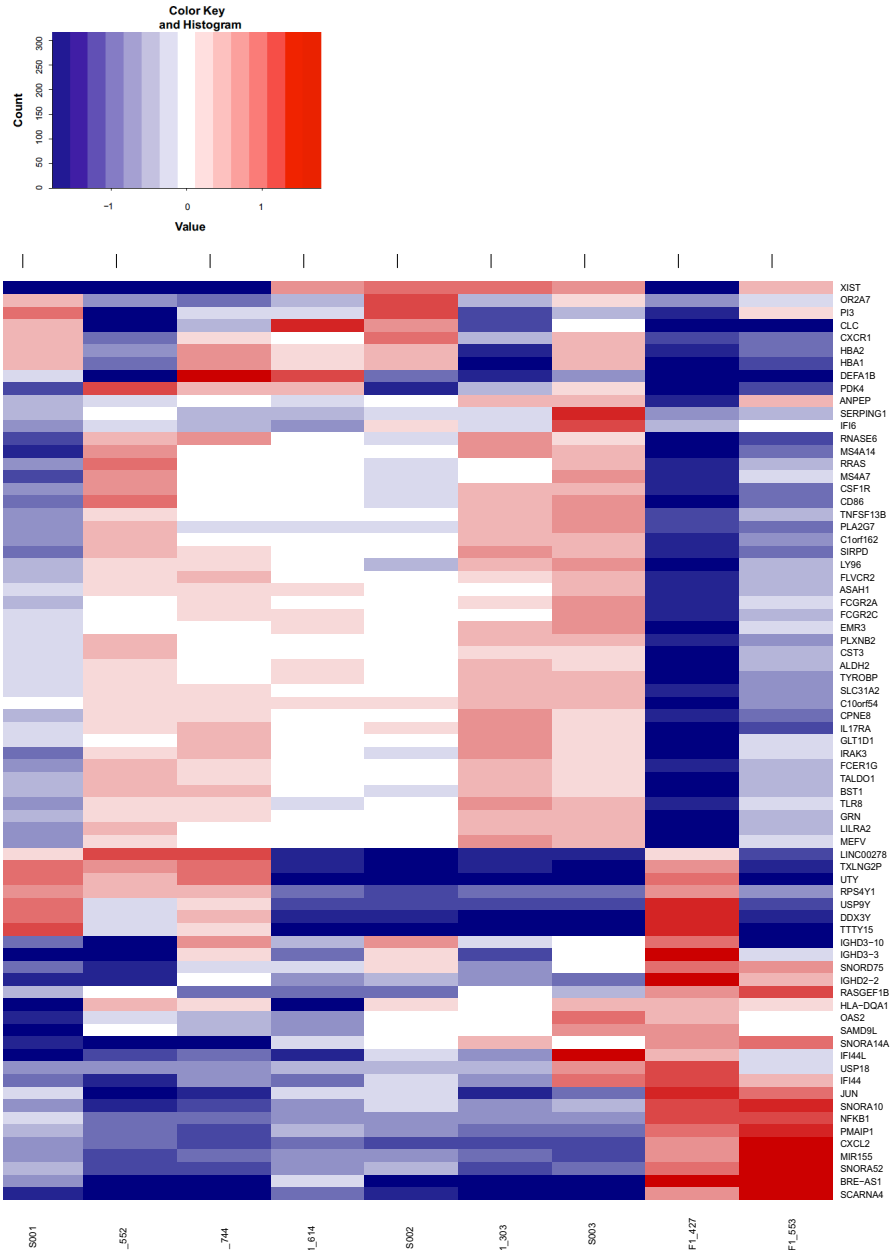

Figure 3. *Gene expression profile NF1_427 versus healthy control (FC>2). NF1's patients has a -2<FC<2. From the left S001 (heathy), NF1_552 (father), NF1_744 (NF1's patient), NF1_614 (NF1's patient), S002 (healthy), NF1_303(NF1's patient), S003 (healthy), NF1_427 (son) and NF1_553 (mother).*

The NF1_552 (father) and NF1_553 (mother) displayed different gene profiles. As second step we focused our attention on the family gene expression. The comparison between NF1_427 vs NF1_553 (83 genes) and NF1_427 vs NF1_552 (726 genes) evidenced the different condition between the child and their parents.

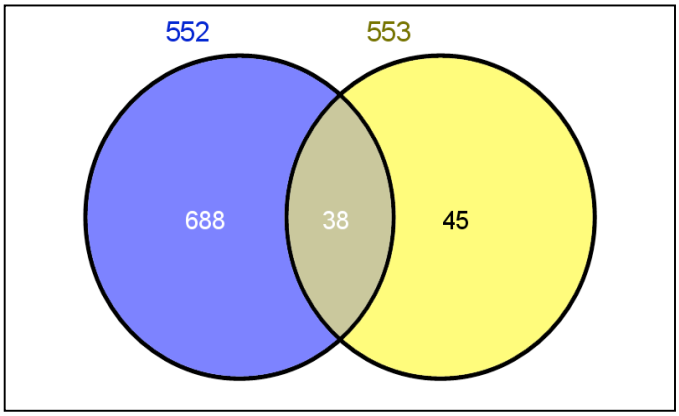

Figure 4. *Venn graph of genes differently expressed in the family (FC >1,58).*

The Venn graph underlined that 38 genes were different expressed in the family (FC=1.58 o 3). The correspondent heat map showed, another time, that NF1_427 had a unique profile.

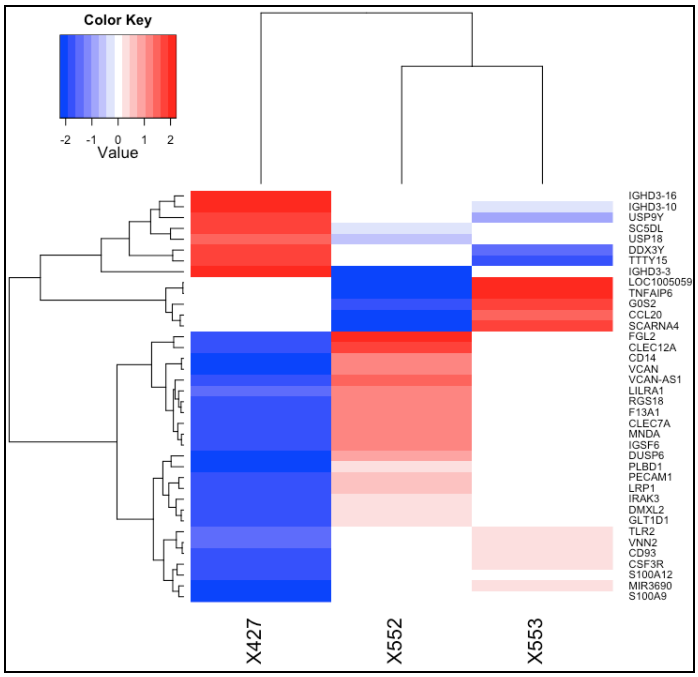

Figure 5. *Heat map of the common 38 gene differently expressed in the family members 427 (son), 552 (father), 553 (mother).*

These 38 genes were implicated in different pathway with no apparently strong correlation for RAS-pathways. Further studies and pathways association were needed to individuate new candidate genes that could help to underline new mechanism implicated in the phenotypic variability typical of neurofibromatosis.

Protein Structural Analysis

Neurofibromin is a large protein of 2818 aminoacids. The NF1 protein is a "tumor suppressor", an activator of Ras-GTPase [9, 10]. Due the large size and the critical role of Ras-GAP domain, this domain is well characterized also at crystal structure. Instead, the two mutations identified in the family, p.Ser2414Cys and p.Gln2665Arg, were in the

CTD domain (C-Terminal Domain) which three-dimensional docking model were not available. However, we took advantage of guidelines reported by Bell and colleagues in 2007 for the interpretation and prediction of structural changes caused by our unclassified variants (UVs) [11]. This methods permitted us to characterize *in silico* the tolerant effect of the NF1 p.Ser2414Cys and p.Gln2665Arg substitutions. Both the variations, separately studied, totaled a score of UV4: possible value of pathogenicity (Table 1).

In details, these guidelines took advantage from Align-GVGD, Polyphen, SIFT, NetPho software and dbSNP database (see materials and methods). The p.Ser2414Cys variant scored higher values of pathogenicity than p.Gln2665Arg among the bioinformatics tolls. The Serine/Cystein change was not present in the dbSNP database. Polyphen and SIFT referred to sequence alignment consider probably damaging and intolerante the amino acid substitution due the evolutionary conservation and physiochemical differences. Moreover align-GVGD classified the variations as most likely to interfere (maximum class: C65).

The Glutamine/Argynine change was either no present in dbSNP database. *In silico* tolls revealed that possibly damaging effect and the intolerant of this variation cause by physiochemical differences and evolutionary conservation of the 2665 Glutamine amino acid, even though GVGD prediction assigned it in to class 35 (intermediate class). Both the variants didn't disrupt any phosphorylation site or functional domain.

Prediction	Ser2414Cys	Gln2665Arg
SIFT	Intolerant (0,02)	Intolerant (0,02)
GVGD	C65 (GV:0.00 GD:111.67)	C35 (GV:0.00 GD:42.81)
Polyphen-2	Probably damaging (0,14 – 0,99)	Possibly damaging (0,81 – 0,94)
Domain	No one	No one
NetPho	No phosphorylation change	No phosphorylation change
dbSNP/EVS	Not present	Not present

Table 1. *In silico prediction results for the two amino acids changes.*

Functional Studies

Later, we conducted few preliminary studies to investigate the functional activity of neurofibromin in the family. I*n vitro* studies were conducted in immortalized lymphocyte (lymphoblast lines) of the family members, the 3 NF1's patients. Neurofibromin is a RAS GTPase activating protein (GAP) which turn off RAS by catalyzing RAS-mediated GTP hydrolysis [12]. Indeed, the active form of Ras binds and activates the kinase Raf and phosphatidylinositol 3-kinase (PI3K), which then sets off a kinase cascade, culminating the

activation MAPK and PI3K pathways. Some of these signals are then transmitted to the nucleus, regulating the expression of genes controlling cell proliferation, cell death, differentiation and migration [13]. For these implications, it has been established that constitutively active mutations of Ras are frequent and associated with multiple human cancers as a result of permanent stimulation of the Raf–MAPK and/or PI3K signaling cascades that lead to uncontrolled cell proliferation and escape of apoptosis [14].

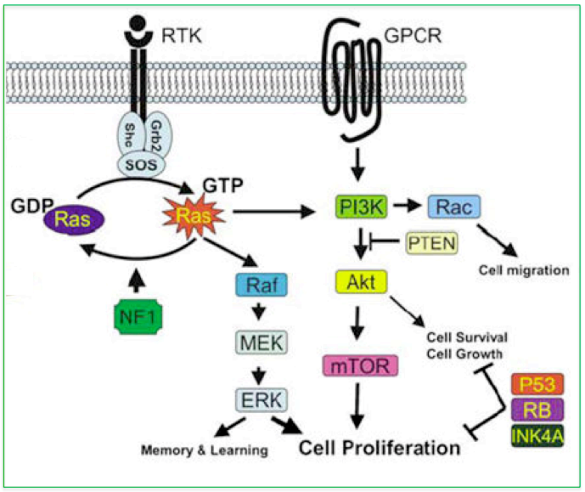

Figure 6. *RAS downstream pathway (adapted from* [13]).

Therefore, we studied the expression level of neurofibromin and we detected the status of the two principal downstream pathways of RAS activity: ERK/MAPK and PI3K/AKT pathways.

First of all, we conducted an ELISA assay (Millipore #17-497) to detect the presence of activated Ras. The assay works on the principle that Ras only binds to its downstream kinase, Raf-1 (MAP Kinase Kinase Kinase), when in its active-GTP bound state. Ras activity

assay underlined a NF1_427's (son) trend to have more RAS active status than his parents (552-553) with similar level of NF1's patients. No statistical difference was obtained.

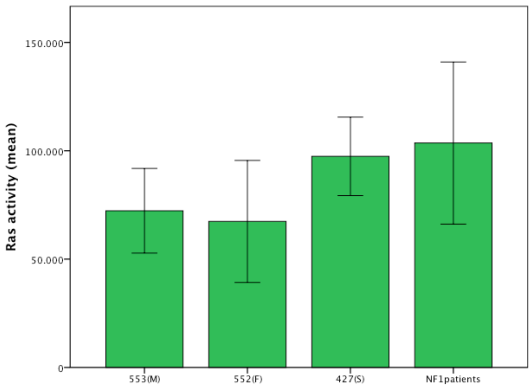

Figure 7. *Elisa assay for RAS activity. Results were representative of two experiments.*

This data was also observed by western blot analysis (Figure 8). Lymphoblast lines showed different level of NF1 expression with a decrease level in 427 sample. Moreover, the activation status of RAS downstream pathways (ERK/MAPK and PI3K/AKT) was more activated in NF1 patients versus the family. In particular, the activation status was distinguished by pERK1/2 and pAKT signals.

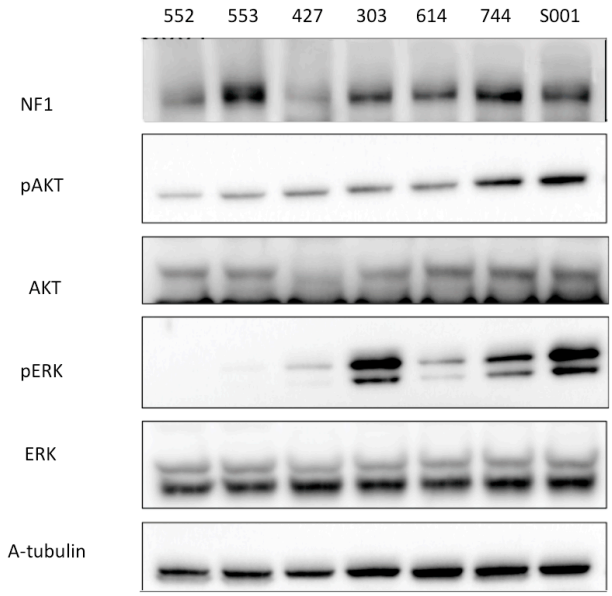

Figure 8. *WB analysis of Lymohblastoid lines*

DISCUSSION

At the age of 2 years old, a boy (427) with macrocephaly, mild psychomotor retardation and four Café-au-lait spots, was submitted to the attention of our diagnostic laboratory for *NF1* gene testing. We performed a DNA-based protocol and we detected two novel missense variations located in the 40 and 46 exons: c.7240 A>T (exon 40) and c.7994A>G (exon 46).

The distinction between a pathogenic and rare benign variant is of utmost importance when mutation analysis is performed as clinical testing and this distinction is, apart from silent or deep intronic sequence changes, particularly challenging for missense alterations.

In the absence of standard functional assay, rigorous criteria must be applied before a novel missense alteration be classified as causing

mutation in order to avoid diagnostic errors. The missenses were not restricted uniquely to any of the so far more characterized regions with known or putative functions (GRD, CSRD, Sec14). In this chapter we proposed a method for the evaluation of missense alterations in a very curious family case (figure 9).

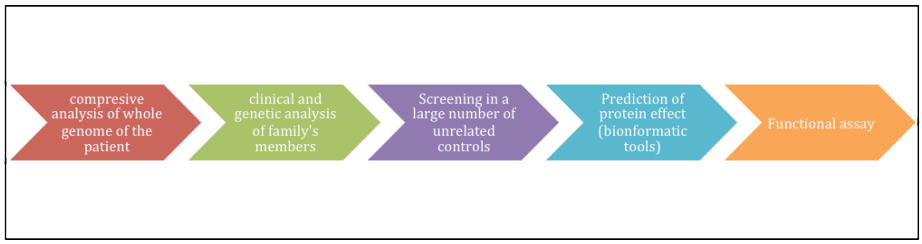

Figure 9. *Schematic method to assign pathogenic value at unknown variant.*

As a first step, after DNA alterations detection, we performed an RNA-based mutation analysis to evaluate possible splicing effect due the missense mutations. We confirmed the simple nucleotide change for both alterations without any splicing effect. Then, as second step, we performed a clinical and molecular genetic assessment of the relevant family members. In familial patients, the missense mutation needs to be proven to segregate with the disorder in the family by analysis of at least one affected relative. The latter argues that missense alterations segregates with the symptoms in the family, but does not provide in itself proof of its pathogenicity. Surprisingly, we detected the c.7240 A>T alteration in the NF1_427'mother (NF1_553) and the c.7994 A>G in the NF1_427's father (NF1_552). An accurate clinical investigations point out partial NF1 features for both the individuals without fulfilling NIH criteria [1]. The father displayed only three neurofibromas, while the mother mother showed one CAL spot

and had difficulties in reading at scholar age. Of note, the mother has a sister who had 2 CAL spots, mild scoliosis and small iris nodules at the ophthalmological examination (NF1 clinical features). No more information was obtained from other members of the family.

As third step we evaluated the absence of the sequence alterations in a large number of unrelated control samples. Also in this analysis we couldn't exclude any of the two variations as possible causative. No one of the 140 healthy controls displayed the same variations.

At this point (four step), we performed bionformatics analysis to predict if the missense alterations can be considered deleterious at the protein level. The two alterations (p.Ser2414Cys and p.Gln2665Arg.) were located in the CTD domain which three-dimensional docking model were not available. Therefore, we took advantage of some bioinformatic tools to evaluate the possible alterations in amino acids that have been conserved over a long evolutionary distance in the *NF1* orthologs (PolyPhen) and algorithms that differentiate one variant from the wild-type at a given codon based on chemical differences (such SIFT or Grantham score GVGD). Following guidelines reported by Bell and colleagues in 2007 for the interpretation and prediction of structural changes caused by UV, we concluded that both the alterations have a possible value of pathogenicity [11]. In particular, the p.Ser2414Cys change obtained higher value in the deleterious prediction (table 1) and it could also confirmed by the clinical history of the mother's family.

Moreover, more molecular and functional features were needed to assign the pathogenicity value. To this purpose we evaluated gene

expression analysis on RNA samples of the family member (mother-father-son), three NF1' patients (with RAS-GRD mutation) and three controls. HTA 2.0 microarray underline the high potential informative results: NF1_427's gene expression profile was unique in comparison to healthy control and NF1's patients (figure 2-3). Gene ontology were classified in intrinsic pathway for apoptosis, metabolism, generation of second messenger molecules and MAPK targets/Nuclear events mediated by MAP kinases. Further studies were needed to figure out new possible intrinsic molecular mechanisms that regulate the phenotypically highly variable disorder in patients with the same alterations. To date, most mutations reported were unique to single families. Therefore, attempts to link the effect of specific mutations with NF1 phenotypes are hampered both on clinical and molecular grounds [15]. Multiple allelism of the NF1 locus, however, cannot be considered to be the main reason for the variability because it doesn't explain the intrafamilial variation. The possible modulation of clinical expression of NF1 by modifying genes were proposed by Easton et al. [16], whereas Ricciardi [17]favors stochastic events as the most important ones. Following this idea, we compared gene expression profile of the three members our family: the son profile were more similar to the mother (83 genes differently expressed) than the father (726 genes). A lot of identified genes list in immunological gene otology probably due the tissue of origin (peripheral lymphocyte). More stringent and selective filters will be applied for further analysis in the future. In addition, further study will be conduct on the 38 genes differently expressed in common in the three family members. Few of these genes, as PECAM, had been already studied in association with

neurofibromin to better characterize the embryonic development and microenvironment contribution in neurofibromas formation [18].

Functional assay were also performed *in vitro* to evaluate the status activity of NF1: western blot analysis of RAS downstream pathways (figure 8) and its direct quantification by ELISA assay (figure 7) showed that NF1_427 display a status more similar to NF1's patients with fulfilling clinical criteria than their parents with a mild clinical features.

We could not completely exclude a bias induced by EBV immortalization. As next we would replicate experiments in lymphocyte of patients to verify the reliability of our *in vitro* model. Lymphoblast model could offer material for protein studies of patient whom mild clinical phenotype and unknown variants coexist. Usually, for these patients no biopsies were available to further studies in fibroblast and schwann cells that are the most studied cell type for their implications in neurofibromas formation. Moreover, the rapid growth allowed us to collect samples for RNA analysis and protein as we tried to set up.

In the future we plan to investigate also the possible role of the two missense mutations in NF1 in migration and memory defects [19, 20]. As the RAS GAP domain constitutes only a small portion of the total protein (almost 13%), it has been suggested that other non-RAS associated functions might exist. To this end, studies in Drosophila have shown that neurofibromin can regulate the cAMP pathway by regulating adenylyl cyclase activity [21, 22], although the molecular mechanism by which this occurs remains elusive [23].

CONCLUSION

We set up a comprehensive analysis for the deleterious predictions for the two novel missense mutations. We could not exclude that both the mutations has a deleterious effect on NF1 protein. Up to date NF1's heterozygous compound were describe only in *cis* positions [15, 24, 25], and homozygous mutant mice for NF1 gene (in RAS-GRD domain) display a variety of development abnormalities that lead to midgestation lethality [26, 27]. The CTD position and the not RAS-GRD function disruption may explain the mild phenotype and the co-existence of two mutations in a *trans* heterozygous compound. Although our hypothesis needs to be sustained by other similar cases, this may illustrate the importance of a re-evaluation of the functional significance of NF1 variants that do not cause an overt Neurofibromatosis 1 phenotype, extending the mutation screening to all long gene, addressing genotype-phenotype correlation studies that will ultimately improve genetic counseling and molecular diagnosis of Neurofibromatosis type 1.

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Chapter 5

SUMMARY

In this study, we present the experience of the Istituto Neurologico Besta after ten years of diagnostic activity of type 1 neurofibromatosis. We found a high mutation rate and the lack of hot spots in the NF1 locus. We analyzed 452 unrelated individuals and we identified 187 different single mutations. Novel mutations, never described in literature, represented the 53% (102/187). 90/102 were small changes (88%), mostly small deletions and insertions (43% - 45/102) often causing frameshifts and premature stop codons. Mutations affecting splicing (21% 21/102), nonsense (11% 11/102) and missense (13% 13/102) mutations were less frequent. Microdeletions and insertions/ deletions of one or more exons represented the 12% (12/102). These mutations were identified by a protocol based of DNA analysis by MLPA and DHPLC, with a mutation discovery rate of 70% (210/297 in cases fulfilling NIH criteria for NF1 diagnosis).

Trying to improve our diagnostic potential we recently developed a multi-step, integrated genetic protocol using MLPA analysis and RNA-sequencing. In the present study we reported the results of this RNA-based protocol in a cohort of 28 blood samples from NF1 patients with complete NF1 features. We detected mutations in 26 of 28 analyzed patients, raising to 93% our discovery rate: 5 mutations

were not detected in a previous screening with DNA-based protocol. These results prompted us to change our routine protocol to the new one based on RNA sequencing.

We also describe a peculiar case of compound heterozygosity in an Italian family. A two year child with clinical phenotype partially fulfilling criteria for NF1 diagnosis displayed two novel missense mutations in exons 40 and 46. Family's studies support the transmission of each genetic variant from the parents. In addition, both parents showed few but typical NF1's features as Cafè-au-lait pigmentation and neurofibromas. The two novel missense variants induced amino acid changes at C-terminal domain (CTD). Molecular and protein characterizations, and the use of bioinformatics tools, suggested, but could not formally prove the pathogenic role of both variations. This was the first case of NF1 compound heterozygosity described in literature.

CONCLUSION

Neurofibromatosis type 1 (NF1) is a rather common autosomal dominant tumor predisposing syndrome that is caused by the germline mutations in the *NF1* gene, one of the human genes with a higher mutation rate. The penetrance of NF1 is full by adulthood, but the severity of the disease varies greatly even among family members carrying the same mutation [1]. In this study we showed the mutational spectrum of NF1 gene in the Italian population. We confirmed the lack of hot spots along the 350kB gene locus and the high mutational rate with the identification of 187 different mutations. Moreover, only 24/187 were recurrent in more than 1 unrelated patient and mutations were mainly *de novo* (60% of sporadic cases).

Strategies for mutation detection and their efficiency have significantly improved during the last decade. many clinical genetics laboratories working on NF1, however, cannot afford expensive mutation detection strategies; therefore, a significant percentage of sporadic NF1 germline mutations remain undetected [2]. Notably, we set up an RNA-based method that helped us to obtain a 20% increase of our previous mutation detection rate.

The new technologies such as next generation sequencing may increase the detection of new alterations in less time. The high potential number of mutations, the high variable phenotypic expression and the higher risk of tumor development in NF1 patients require that laboratories share data through the submission of new mutations and correlated clinical data, in open databases such as LOVD and HGMD, thus helping to establish the diagnosis in patients

who present with atypical manifestations or unusual combination features.

The wide variability of the phenotypic expression even among affected members of the same family highlights the complex relationship between genotype and phenotype of patients. A comprehensive exam was needed to analyze two novel missense mutations (c.7240 A>T and c.7994 A>G). Bioinformatics predictions and functional assay in GRD domain revealed the possible coexistence of two mutated alleles in NF1 gene, even if they didn't hit the GAP Ras domain. Moreover, data obtained by gene expression analysis confirmed the high complexity of neurofibromatosis, even inside a family, and the possible contribution of modifier genes affecting genotype/phenotype correlations [3-5].

FUTURE PERSPECTIVES

Cafè-au-lait macules are often the first signs of NF1 and may already be present at birth, increasing in number during the first year of life. However, waiting for more symptoms to appear in order to ascertain the diagnosis on a clinical basis can be very stressful for families. Making a molecular diagnosis as early as possible will become even more important as better therapeutic interventions become available. In the future we plan to implement our diagnostic activity by the introduction of Next Generation Sequencing techniques [6]. In particular we plan to design a panel for the principal diseases resembling NF1: Legius Syndrome (*SPRED1* gene), Schwannomatosis (*SMARC1* and *LZTR1* genes) and also neurofibromatosis type II (*MERLIN* gene) [7-9]. Neurofibromatoses and Legius syndrome exhibit a unique phenotype, but owing to the common mechanisms of RAS/MAPK pathway dysregulation, they share many overlapping characteristics and an increased cancer risk [7]. Moreover, NF1 variable phenotype and existing subtype as segmental or mosaicism NF1 increase the phenotype overlapping.

The molecular diagnosis is relevant since it might impact on the treatment of the individual, and is also important when it comes to family planning and genetic counseling. Nowadays, the therapy of NF patients is the surgical removal of neurofibromas and the treatment of disease-associated clinical features. There are some experimental results and drug developments with growth factors and tyrosine-kinase and oncogenic kinase inhibitors [10-12]. In case of the best

efficient application of these medications, it is important to know the exact and detailed genetic background of each NF patient. Also in our Institute a pilot, clinical trial using gleevec for plexiform neurofibromas is ongoing in 6 patients.

Finally, we would further characterize the clinical features of patients in our ten year database with a particular focus on the 102 new mutations. As mentioned before, to share molecular and clinical data through their submission in open databases is crucial for a syndrome with high mutational rate and with atypical clinical manifestations or unusual combination features. We will also focus on the huge amount of data generated by gene expression profiling of the family case of compound heterozygosity trying to find modifier genes that could contribute to the peculiar clinical/molecular features associated to the missense mutations.

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