A Mild Neurofibromatosis Type 1 Phenotype Produced by the Combination of the Benign Nature of a Leaky NF1-Splice Mutation and the Presence of a Complex Mosaicism

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ABSTRACT: Here we analyze the genetic and molecular basis responsible for a very benign phenotype observed in an NF1 patient. Quantification of cells carrying the NF1 mutation in different samples derived from the three embryonic layers revealed mosaicism. Furthermore, the construction of a minigene with patient’s mutation (c.3198–314G > A) confirmed its benign nature due to the leakiness of the splicing mechanism that generated a proportion of correctly spliced transcripts. Hence, we concluded that the mild phenotype observed in this patient is the result of the presence of mosaicism together with the benign nature of a leaky NF1-splice mutation. Finally, with the aim of developing a personalized therapeutic approach for this patient, we demonstrated correction of the splicing defect by using specific antisense morpholino oligomers. Our results provide an example of the molecular complexity behind disease phenotypes and highlight the importance of using comprehensive genetic approaches to better assess phenotype–genotype correlations.

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KEY WORDS: NF1; mosaicism; genotype-phenotype; splicing

Neurofibromatosis type 1 (NF1; MIM# 162200) is an autosomal dominant disorder characterized by an increased predisposition to develop certain types of malignancies as well as by the presence of a wide range of clinical traits involving cells of neural crest origin (reviewed in [Riccardi, 1992]). NF1 is caused by germline mutations in the NF1 gene, which is one of the human genes with a higher mutation rate. Comprehensive genetic studies identified more than 1,100 disease-causing mutations allowing a precise depiction of the NF1 germline mutational spectrum (reviewed in [Messiaen and Wimmer, 2008]). So far, two constitutive NF1 mutations have been correlated with a particular NF1 phenotype. Individuals with type 1 NF1-deletions, which encompass 1.4 Mb of genomic DNA [Kayes et al., 1994; Lopez Correa et al., 1999] and involve several other genes in addition to the NF1, are characterized by a severe phenotype, consisting of learning problems, dysmorphic features and a high number of dermal neurofibromas [Mautner et al., 2010; Pasmant et al., 2010]. By contrast, patients with the recurrent c.2970–2972delAT mutation seem to express a moderate phenotype characterized by the absence of dermal neurofibromas [Upadhyaya et al., 2007].

It has been suggested that a proportion of the new mutations are actually somatic implying than some sporadic patients are mosaics for a NF1 mutation [Zlotogora, 1993; Kehrer-Sawatzki and Cooper, 2008]. Depending on the stage during development of the occurrence of the mutation we can distinguish patients showing generalized mosaicism, segmental mosaicism, and gonadal mosaicism [Ruggieri and Huson, 2001]. Generalized mosaicism cases exhibit typical symptoms of the disease in a mild generalized form, making them very difficult to distinguish from nonmosaic patients. Segmental manifestation show clinical manifestations limited to one or a few areas of the body [Crowe et al., 1956; Moss and Green, 1994; Riccardi, 1982]; this is a rare condition that occurs at around 1:36,000–40,000 individuals [Friedman et al., 1999; Ingordo et al., 1995; Ruggieri and Polizzi, 2000; Wolkenstein et al., 1995]. Gonadal mosaicism is confined to the germline and is extremely uncommon in NF1 [Bottilo et al., 2010; Lazaro et al., 1994]. Identification of somatic mosaicism and assessment of tissues affected by the NF1 somatic mutation is difficult and represents a challenge because it is especially important for providing accurate genetic counselling to the patient.

For several genetic conditions, genotype-phenotype studies have suggested the importance of mechanisms regulating splicing as modifiers of phenotype in carriers of splicing defects [Nissim-Rafinia and Kerem, 2005]. For instance, a leaky effect of some splicing mutations associated with the production of wild-type transcripts from mutated alleles has been described. In some cases this phenomenon has been associated with a mild phenotype [Beck et al., 1999]. The high number and diversity of splicing
mutations in the *NF1* gene made it interesting to explore the occurrence of leakiness in the splicing mechanism and its putative relation to the severity of the disease.

In this work we describe the molecular basis underlying the mild NF1 phenotype of a patient fulfilling the NIH-NF1 established diagnostic criteria. Written informed consent was obtained from the patient following our institutional review board approved protocol. The patient is a 34-year-old woman who is a sporadic case of the disease (Fig. 1A, left panel) showing 20 café-au-lait spots on the trunk and upper extremities, mild scoliosis, axillary and submammary freckling, and presence of less than 50 minuscule neurofibromas (a few millimeters in diameter) located on the trunk, which started to appear when she was 18 years old (Fig. 1A, right panel). The patient does not have Lisch nodules, any dysmorphism or learning disability. The *NF1*-mutational analysis using DNA isolated from peripheral blood of the patient revealed a point mutation in intron 19a of the gene (c.3198–314G > A) (Supp. Table S1). This mutation is a deep intronic mutation that creates a new cryptic acceptor splice site that uses two different cryptic donor splice sites present in the wild-type sequence (Supp. Table S2), to generate two aberrantly spliced transcripts showing inclusion of two different cryptic exons (Fig. 1B); both cryptic exons would generate the same putative truncated protein (p.Asp1067TrpfsX7).

We investigated two possible biological mechanisms that could explain the mild phenotype of the patient: somatic mosaicism and presence of mild *NF1*-mutation. To explore mosaicism we used a quantitative approach, based on the analysis of a single nucleotide primer extension reaction (SNaPShot analysis), to determine the proportion of cells containing the mutated allele in a subset of samples representative of the three embryonic layers (Supp. Materials and Methods). As this *NF1* mutation had never been described before in other patients, it was impossible to obtain genetic material representative of a bona fide heterozygous sample that could be used as a control for quantification. Hence, we generated artificial controls consisting of two plasmids; one containing the patient’s mutation and the other bearing the wild-type sequence (Supp. Materials and Methods). A mixture of serial proportions of both plasmids was used to obtain a standard curve that allowed estimation of the proportion of mutant alleles present in the different tissues obtained from the patient (Supp. Figs. S1 and S2). This analysis revealed that samples derived from the endoderm and mesoderm, such as uroepithelial cells and peripheral blood, respectively, showed a proportion of mutant allele of around 50% (Fig. 1C and Supp. Fig. S3), indicating that all the cells from these tissues are carrying the mutation in heterozygosis. However, samples derived from the ectoderm, such as skin, buccal swab or hair roots, showed a mutant allele proportion lower than 50%, ranging from 20 to 35%, suggesting that only a proportion of the cells of these tissues were carrying the *NF1* mutation. Cells from saliva showed an intermediate value that was in agreement with its nature, consisting of a mixture of white blood cells (mesoderm) plus buccal epithelial cells (ectoderm). To validate our results and confirm that the observed different proportions of mutated and WT alleles in the SNaPShot analysis were reflecting a somatic mosaicism and were not caused by amplification artefacts due to the different nature and origin of DNAs analyzed, we performed a control SNaPShot analysis using a SNP unrelated to the disease in the same set of DNA samples. We studied SNP rs2075786 located in an intronic region of the TERT gene, located on a different human chromosome and for which our patient was heterozygous. In this case, the proportion of both alleles was close to 50% in all tested DNA samples (Fig. 1C and Supp. Fig. S4), a result that reinforced the validity of our previous results observed in the SNaPShot analysis of the *NF1* mutation. Taking all our results together we can conclude that the patient studied here is a case of a NF1 mosaicism as the *NF1* mutation is present in different proportions in different cell types. Hence, this *NF1* mutation may have occurred early during development; as cells derived from the three embryonic layers are carrying the mutation in contrast to cases of segmental mosaicism, where the proportion of mutated cells in nonneural crest derived tissues can often lie below the detection level of routine analysis [Maertens et al., 2007]. Although several cases of mosaicism have
been described for NF1, the role of mosaicism in NF1 is still scarcely analyzed and limited to few number of cases [Kehrer-Sawatzki and Cooper, 2008]. The use of different methodological approaches with high sensitivity such as SNaPShot analysis or real-time quantitative allele discrimination [Aretz et al., 2007; Maertens et al., 2006], together with the investigation of several different tissues in cases where a mild form of the disease is observed, will help to ascertain the role of somatic mosaicism in Neurofibromatosis type 1.

Taking into consideration the embryological origin of the different tissues analyzed and the fact that the majority of NF1 traits have a neural crest-derived cell origin (reviewed in [Raedt et al., 2008]), we were surprised by the proportions of mutated cells identified, greater in tissues mainly derived from the endoderm or mesoderm and smaller in tissues derived form the ectoderm. We decided to further explore the complex mosaicism exhibited in this patient by performing an X-chromosome inactivation (XCI) assay in the same set of tissues (Supp. Materials and Methods) [Allen et al., 1992]. X-chromosome inactivation is a stochastic event that occurs in the early stages of embryonic development in female embryos [Lyon, 1961, 1962]. If a genetic mutation occurs after this inactivation, one can perform an X-chromosome inactivation test in different tissues in order to ascertain whether any bias is observed in the proportion of inactivation in both X-chromosomes and compare these results to the proportion of cells carrying the given genetic mutation [Wang et al., 2009]. In our case, determination of clonal expansion was based on the analysis of DNA methylation and CAG tandem repeats at the human androgen receptor locus (HUMARA) [Allen et al., 1992], located on chromosome X, in the same tissues were mutation analysis was performed (with the exceptions of skin and hair roots). The number of CAG repeats differentiated the parental X chromosomes and methylation status distinguished the active and inactive X chromosome. In the absence of proliferative differences (advantages or disadvantages) between NF1-mutated and nonmutated cells, completely random XCI would be expected to result in around 50% inactivation of each X chromosome in all tissues. By convention, mildly skewed XCI was defined by an allele ratio 80–90% inactivation, and extremely skewed XCI was defined by an allele ratio >90% inactivation [Beever et al., 2003; Kimani et al., 2007]. Interestingly, we observed skewed XCI in the case of peripheral blood, saliva and uroepithelial cells with ratios of 12.88; 15:85, and 10:90, respectively. None of the rest of tissues from the patient, or a subset of control blood DNA samples, showed skewed XCI (Fig. 1D and Supp. Fig. S5). Tissues carrying the highest proportion of mutated cells coincided with those exhibiting skewed XCI. Moreover, in all these tissues, the same X chromosome was predominantly inactivated, making it unlikely that this observed skewed X-inactivation was the result of a purely random process. These results suggest a proliferative advantage of certain cells carrying the NF1 mutation that results in higher proportions of both percentage of mutated cells and cells with skewed X chromosome inactivation. Consequently, these proliferative differences suggest that the observed proportions of mutated cells in the adult tissues analyzed in the present study do not reflect the initial percentage of mutated cells in the different embryonic cell layers. However, these results have to be taken carefully as it has been reported that X-inactivation ratios may vary between different tissues within one normal individual [Sharp et al., 2000].

To investigate a second possible cause of the observed mild NF1 phenotype, the benign nature of the NF1 mutation, we analyzed and quantified the expression of mutant transcripts produced by the deep intronic NF1 mutation identified, exploring different tissues from the same patient. We analyzed any deviation from the expected 50:50 proportion of mutant versus normal transcripts, taking into consideration the proportion of mutated cells identified in the analysis of DNA from different tissues. The analysis of RNA from fresh tissues (Supp. Materials and Methods) indicated, with a certain degree of variation between samples, a low proportion of mutated transcripts, ranging from 1.4% to 14.41% and none in the hair root sample (Fig. 2A, upper panel). The study of cell cultures (lymphocytes and fibroblasts) (Supp. Materials and Methods) revealed a low proportion of mutated transcripts as well as the action of the nonsense-mediated mRNA decay (NMD) mechanism on mutated-mRNA, because puromycin treatment was able to increase the levels of mutant transcripts observed in both cell types (Fig. 2A, bottom panel). By comparing the proportion of mutated transcripts (Fig. 2A) with the percentage of mutated DNA (Fig. 1C) in the same tissues, it became clear that there was a reduction in the proportion of mutated RNA. An illustrative example was the analysis of lymphocyte cells that showed equal proportions of mutated and wild-type alleles at the DNA level. However, the analysis of their transcriptional profile provides evidence of a much lower proportion of aberrantly spliced transcripts than the expected 50% (20% after puromycin treatment and less than 10% without this treatment). Altogether, the differences between observed and expected proportions of aberrantly spliced transcripts, even after avoiding NMD, suggested the possibility that wild-type transcripts were also produced from the mutated allele, resulting in a low proportion of abnormal transcripts. In order to confirm this hypothesis we also constructed a minigene carrying the mutated allele (Supp. Materials and Methods). The analysis of the transcripts generated by the minigene containing mutation c.3198–314G>A indicated the production of both mutated and wild-type transcripts, while the minigene encoding the normal sequence only produced normal transcripts (Fig. 2B and Supp. Fig. S6), confirming that the low proportion of mutant transcripts is due to the production of normal transcripts from the mutated allele due to leakiness of the splicing mechanism. Therefore, the particular benign nature of this NF1 mutation was possibly contributing to the mild phenotype observed in our patient, by acting as a hypomorphic allele rather than a null one.

Finally, and with the aim of starting to design personalized therapeutic strategies for NF1 patients, three different specific AMOs blocking cryptic splice sites used by the mutation were designed (as previously reported) [Pros et al., 2009]. AMOs were designed, synthesized, and purified by Gene Tools (Philomath, OR) and endo-porter (GeneTools) was used to deliver AMOs into skin-derived fibroblasts from the patient (Supp. Materials and Methods). We observed that the three designed AMOs were able to reduce the levels of mutant transcripts, although a complete correction was only observed when a combination of the three AMOs was used (Fig. 2C, upper panel) as has also been described in other genetic disorders [Gurvich et al., 2008]. To confirm that this reduction was specific to the AMO designed we performed the same treatment but using an unspecific AMO, designed to block a donor splice site generated by a different mutation located in intron 3 of the NF1 gene (Supp. Materials and Methods). As expected, no effect on the proportion of mutant transcripts was observed when using an unspecific AMO. Furthermore, we observed that IVS19a-AMO donors 1 and 2 inhibit, in a specific manner, the two aberrant transcripts generated. IVS19a-AMO donor 1 preferentially inhibits aberrant transcript 1 (r.3197–3198ins3198-214-3198-312) whereas IVS19a-AMO donor 2 preferentially inhibits aberrant transcript 2 (r.3197–3198ins3198-245-3198-312) (Supp. Fig. S7). Finally, to confirm that correction of aberrant splicing by AMO treatment at
RNA level had some effect at a functional level, we indirectly assessed neurofibromin function, by measuring levels of active Ras (Ras-GTP) as an indicator of neurofibromin GTPase activity (Supp. Materials and Methods). We treated primary fibroblast cultures carrying the deep intronic mutation first with one AMO blocking the newly created acceptor splice site and second with a combination of three AMOs that were designed to block all cryptic splice sites located at intron 19a. We found that levels of Ras-GTP were lower in fibroblasts treated with specific AMOs than in untreated fibroblasts or in fibroblasts treated with an unspecific AMO (Fig. 2C, bottom panel), in agreement with our previous results using cell lines derived from other NF1 patients carrying the same type of mutation [Pros et al., 2009]. This decrease in active Ras levels suggests that AMO treatment was indeed restoring neurofibromin GTPase function.

To conclude, in this report we are presenting a very illustrative case where a combination of different biological processes such as somatic mosaicism and the leaky nature of a splicing mutation, are the possible causes of the mild NF1 phenotype observed in our patient. Our results highlight the complexity of genotype–phenotype correlations and the importance of performing comprehensive genetic studies to interpret clinical findings and facilitate genetic counselling.

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Figure 2. Quantification of transcripts, minigene assay, and AMO treatment. A: RT-PCR analysis of total RNA was performed using specific primers to analyze the three types of transcripts produced (wild-type and two aberrant transcripts). The Y-axis of each graph shows the proportion of aberrant transcripts versus the total. Results are represented by a bar consisting of the mean for at least three independent experiments. Upper panel: results from fresh tissues. Bottom panel: results from cultured lymphocytes and fibroblasts. P, Puromycin. B: Results of the RT-PCR analysis of the two constructed minigenes. WT, corresponds to the minigene carrying mutation c.3198—314A; WT, correspond to the minigene with the normal sequence (c.3198—314G); C—, negative control. C: Correction of NF1 aberrant splicing and restoration of the neurofibromin function by AMOs. Upper panel: RT-PCR analysis shows the proportion of aberrant transcripts versus the total in the Y-axis. Results are represented by a bar consisting of the mean ± SD for at least three independent experiments. C1, control, untreated cells from the patient; A, AMO blocking acceptor splice site, D1, AMO blocking Donor 1 splice site; D2, AMO blocking Donor 2 splice site; A+D1+D2, combined AMOs blocking the three splice sites; C2, unspecific AMO blocking a donor splice site created by a different mutation located in intron 3 of the NF1 gene. Bottom panel: morpholino treatment shows reduction of Ras-GTP levels in fibroblast cell cultures from the patient.
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