# Quantification of *NF1* transcripts reveals novel highly expressed splice variants

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**Abstract Previously, we have shown that the *NF1* gene gives rise to multiple novel splice variants. In the present study, nine *NF1* variants were quantified by real-time PCR in various human tissues. Some of these variants were expressed at low to moderate low levels and possible implications of these findings are discussed. Interestingly, two variants (NF1-ΔE4b and NF1-ΔE43) were shown to be highly expressed in specific tissues. NF1-ΔE43 lacks a nuclear targeting sequence and might be functionally different from full-length *NF1*. These novel *NF1* splice variants might expand our understanding of the role of neurofibromin.** 

*Key words:* Neurofibromatosis type 1; Neurofibromin; Alternative splicing; Real-time polymerase chain reaction; Quantitative polymerase chain reaction

## 1. Introduction

Neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder affecting ~1 in 3000 individuals. NF1 is fully penetrant and is notorious for its tremendous variable clinical expression, even among members of the same family. Although the *NF1* protein product, neurofibromin, is ubiquitously present, the main manifestations are neurocutaneous. Patients present with café-au-lait spots, neurofibromas, iris hamartomas (Lish nodules) and freckling of axillary and inguinal regions. Other manifestations include learning disabilities, optic glioma, bone abnormalities and an increased risk of specific malignancies [1]. The *NF1* gene spans ~350 kb of genomic DNA and produces a 11–13 kb transcript containing 60 exons [2–4].

Alternative splicing is an important mechanism whereby different mRNAs are generated from the same gene, thus increasing the coding capacity. It is often regulated in a tissue- or developmental-specific manner. Several alternative transcripts have been described for the *NF1* gene. Three alternative exons (9br, 23a and 48a) expand the size of the coding region. These isoforms were also detected at the protein level [5,6]. Four other transcripts have been reported (the deletion of exon 29, exon 30, exon 29/30 and the N-isoform) which would lead to truncated neurofibromins [7,8], but no further confirmation at the protein level has been reported yet. Using an RT-PCR plasmid library we determined the extent of alternative splicing in *NF1* [9], and a survey of various new *NF1* splice variants was described. In the present study, further insight into the significance of some of these variants is provided by accurate quantification of variant transcript levels in different human tissues. Isoform-specific quantification using real-time PCR [10] was applied to quantify nine *NF1* transcripts. Using this method, several variants were identified that were highly expressed in a tissue-specific manner, which is very suggestive for a functional significance of these transcripts. In addition, two transcripts were quantified in several NF1 patients.

## 2. Materials and methods

### 2.1. RNA isolation and cDNA synthesis

RNA was isolated from fresh blood leukocytes (pooled from four persons), melanocytes (pooled from two persons), fibroblasts (pooled from two persons) and short-term lymphocyte cultures using TRIzol LS Reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (2 μg) was reverse transcribed using SuperScript II Reverse transcriptase (Invitrogen) and random hexamers (Amersham Biosciences). In addition, tissues were obtained as both commercial cDNA and RNA (brain, heart, kidney, liver, lung, pancreas, placenta, skeletal muscle), allowing us to compare different cDNA and RNA preparations, or only RNA (fetal brain, fetal liver, spinal cord, cerebellum) from Clontech. All commercial cDNAs and RNAs were pooled from various persons. Cytoplasmic RNA was extracted from four fresh lymphocyte cultures using the RNeasy mini-kit (Qiagen). The Oligitex Direct mRNA kit (Qiagen) was used to purify poly($A^+$) RNA from cytoplasmic RNA.

### 2.2. Real-time PCR

Primer design, generation of standard curves and quantification of splice variants was performed as described [10]. In short, boundary-spanning primers were designed that specifically recognize and amplify each splice variant (Table 1), using the Primer Express 1.5 software (Applied Biosystems). Specificity was confirmed using a plasmid containing the full-length transcript as template. The boundary spanning primers, created for amplification of the shorter transcripts, were not able to amplify this plasmid, resulting in the absence of a signal. For generation of the standard curves, PCR products of each spliced and full-length transcript were run on a 3% TBE agarose gel. The fragments were excised and eluted using QIAquick (Qiagen) spin columns. The concentrations of the PCR products were measured using the PicoGreen reagent (Molecular Probes) on a TD-360 fluorometer (Turner Designs). Absolute standard curves were made based on a 10-fold serial dilution series ranging from $2.4\times10^6$ copies to 2.4 copies. Amplification reactions contained 1×SYBR Green I PCR Mastermix (Applied Biosystems) and variable optimized primer concentrations (Table 1). The PCR conditions were 95°C for 10′, followed by 40 cycles of 95°C for 15″ and $T_a$ (Table 1) for 1′. Melting curves were

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*Abbreviations:* NF1, neurofibromatosis type 1; GAP, GTPase-activating protein

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Table 1
Sequences, quantities and annealing temperatures of primer pairs

| Amplicon | Forward primer | Reverse primer | Primer quantity (nM) | Annealing temperature ($T_a$, °C) |
|---|---|---|---|---|
| NF1-Δ4a-2 | agtctttagtcgcatttctaccaggtta | ttgtcttctgaacaaacagttaattcct | 300 | 60 |
| NF1-ex4a-2 | gcatttctaccagtaaccttgatgatac | tctgaacaaacagttaattcctgtaacc | 300 | 60 |
| NF1-Δ4b | cgcatttctaccagaaacagcat | tgctaactgcgcaaccttctt | 100 | 62 |
| NF1-ex33 | cacacatggactggtcattaatatca | tcaaaacttgcttggtctcttcac | 300 | 60 |
| NF1-Δ33 | tgtagctttggcttctggaaatg | caaaacttgcttggtctcttcttg | 300 | 60 |
| NF1-ex29/30 | gcagttctgacccgagtttacg | atgtctctagtaactggccctcgat | 200 | 60 |
| NF1-Δ29 | agacaccaaagtttctattaaagtcagct | ggtacaagttaaggcacacagaagatta | 200 | 60 |
| NF1-Δ30 | tctgacccgagtttacggtattg | agatttgacagccatggagtcat | 200 | 60 |
| NF1-Δ29/30 | caaagacaccaaagtttctattaaagtattg | ggagtcatgtattccaaacaaaggt | 200 | 60 |
| NF1-ex37 | acgagtgtctcatgggcagat | actgttgtaagtgtcaggtccttttaag | 100 | 62, 3% DMSO |
| NF1-Δ37 | cttgttgtctttgggtgtattagca | gcagaggcgagtccttgc | 100 | 62, 3% DMSO |
| NF1-ex43 | tgaggagagtagcagaaactgattatg | cgtaaatgtgggtgctgttgtg | 300 | 62 |
| NF1-Δ43 | gcaaccttctcaggccaaca | ggaaatcctctgagtttcaagca | 300 | 62 |
| NF1-ex45 | aagtctttcctgttgtgcataatttg | caatgataacagggtgttgatcttaga | 250 | 62 |
| NF1-Δ45 | gctgcttcttactgttctagcataattt | taacagggtgttgatcttagagtcca | 250 | 62 |
| mNF1-Δ43 | acagccttctcaggccaaca | ggaatcctttgggtttcaagca | 300 | 62 |
| mNF1-ex43 | tgagaagagtggcagagactgactatg | cgtaaatgtgggtgctgctg | 300 | 62 |
| mNF1+/−ex37 | ctcttgtggtgtttggctgtattagc | ggactcttgtcattgaatatccggag | 300 | 62 |
| mNF1+/−ex4b | atctgccattttcttcacacctgc | ccggataattttctacccagttcc | 300 | 62 |
| mNF1+/−ex43 | aaaccagtccccgagcca | gcaaaccgccataagccatt | 300 | 62 |

generated after amplification. For each experiment, 100 ng cDNA (total RNA equivalent), 0.4 ng Clontech cDNA (mRNA equivalent) or 6 ng cDNA (poly($A^+$) mRNA) were used. Data were collected using the 5700 SDS thermal cycler (Applied Biosystems). Each sample was tested in duplicate. For eight tissues, expression levels could be measured in two independent samples (commercial cDNA or cDNA prepared in our lab from a different RNA sample, see Section 2.1). Data are expressed as the ratio of the copy number for the alternative transcript to the copy number of the total *NF1* expression level (i.e. the sum of the copy numbers of alternative and full-length transcripts).

*2.3. Determination of human–mouse conservation of alternative splicing*

Primers were designed to simultaneously amplify both transcripts (with or without alternative exon) in the same reaction (Table 1). PCR conditions were 35 cycles of 92°C 1′, $T_a$ 50″ and 72°C 1′ on a PTC-200 thermal cycler (BIOzym). The PCR products were cloned in the pCR-2.1-TOPO vector (Invitrogen) and transformed into TOP-10F′ cells. The colonies were lysed and the subcloned fragments were reamplified with the same primers. The length of each clone was verified by agarose gel electrophoresis. Mouse tissues (mouse heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis) were obtained as cDNA from Clontech. In addition, real-time PCR using boundary spanning primers (Table 1) was performed in order to screen for the NF1-ΔE43 variant.

## 3. Results and discussion

*3.1. Quantification of splice variants*

Selection of transcript variants for further study [deletion of respectively exon 4b (NF1-ΔE4b), exon 33 (NF1-ΔE33), exon 37 (NF1-ΔE37), exon 43 (NF1-ΔE43) and exon 45 (NF1-ΔE45)] was based on a semi-quantitative study, whereby PCR products were separated on an ALF-express automated DNA sequencer. In addition, we investigated four splice variants already reported in literature: the insertion of 31 bp between exon 4a and 4b (exon 4a-2, NF1-insE4a-2) [11,12] and the deletion of exon 29, exon 30 and exon 29/30 (NF1-ΔE29, NF1-ΔE30, NF1-ΔE29/30) [7]. To investigate the significance of these variants a real-time PCR assay was developed for accurate quantification of the transcript levels based on specific amplification of *NF1*-splice variants using well-validated boundary spanning primers. Absolute standard curves over a wide dynamic range were generated using a dilution series of fluorometrically quantified PCR products [10]. Expression of the different splice variants was investigated in several tissues (Table 2). Comparison of fetal brain and liver with adult brain and liver expression levels indicates that splicing may be differentially regulated during differentiation in these tissues. Four transcripts show a low expression level ($<1\%$: NF1-ΔE29, NF1-ΔE29/30, NF1-ΔE33 and NF1-ΔE45) of which a few are expressed in a tissue-specific manner. Three are moderately low expressed ($\leq 2.5\%$: NF1-insE4a-2, NF1-ΔE30, NF1-ΔE37) and two exhibit a remarkably high expression in specific tissues (5–22%: NF1-ΔE4b, NF1-ΔE43). Determination of the intra-assay variability on calculated quantities for duplicated samples of all tissues showed a good reproducibility. Median coefficient of variation (CV) for full-length transcripts was 7.2%. PCR reproducibility for very low copy numbers is influenced by distribution statistics (Poisson's law) and small differences in amplification efficiency which can be seen in the larger median CV-value for duplicated samples of the variant transcripts (CV = 19.4%), as some of these are expressed in low amounts. In addition, interassay variability on the calculated percentages of each variant was assessed using two different cDNA samples prepared from the same RNA sample (median CV = 13.6%). Furthermore, the availability of two cDNA samples for eight tissues (commercial cDNA versus cDNA prepared in our lab) derived from different RNA samples gave us the opportunity to compare interassay variability on the calculated percentages between different RNA and cDNA preparations. As expected, the highest level of variability was observed in this last group (median CV = 24.2%).

*3.2. Interpretation of the quantitative data*

The functional significance of the low to moderately low expressed transcripts constituting $<2.5\%$ of the total *NF1* expression level is unclear. Different explanations can be given for this low level of expression. First of all, these low ex-

Table 2
Quantity (in percentage) in various tissues of nine splice variants of the *NF1* gene

| Tissue | Splice variants (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | NF1-insE4a-2 | NF1-ΔE4b | NF1-ΔE29 | NF1-ΔE30 | NF1-ΔE29/30 | NF1-ΔE33 | NF1-ΔE37 | NF1-ΔE43 | NF1-ΔE45 |
| blood | *1.349* | **5.032** | 0.016 | *1.688* | 0.055 | 0.263 | 0.204 | *4.286* | 0.117 |
| fetal brain | *1.287* | *1.493* | 0.099 | 0.078 | 0.125 | 0.091 | 0.039 | 0.754 | 0.086 |
| brain | 0.614 | *1.932* | 0.185 | 0.050 | 0.204 | 0.111 | 0.243 | *2.873* | n.d. |
| heart | 0.527 | *2.269* | 0.080 | 0.462 | 0.709 | 0.465 | *1.074* | *4.298* | n.d. |
| kidney | 0.242 | *1.054* | 0.021 | 0.515 | 0.158 | 0.167 | 0.305 | **11.650** | n.d. |
| fetal liver | 0.395 | *1.901* | n.d. | 0.495 | 0.046 | 0.699 | 0.637 | **21.956** | 0.077 |
| liver | 0.489 | *1.202* | 0.017 | *1.234* | 0.131 | 0.329 | *1.326* | **15.557** | n.d. |
| lung | 0.244 | *1.832* | 0.034 | 0.953 | 0.049 | 0.465 | 0.864 | **6.246** | 0.512 |
| pancreas | 0.265 | *1.540* | 0.063 | 0.216 | n.d. | 0.919 | 0.214 | *2.238* | n.d. |
| placenta | 0.493 | 0.897 | n.d. | 0.920 | 0.397 | 0.402 | 0.478 | **7.952** | 0.043 |
| skeletal muscle | n.d. | *1.741* | n.d. | n.d. | n.d. | 0.498 | *2.111* | **14.307** | n.d. |
| melanocytes | 0.263 | 0.825 | n.d. | 0.456 | 0.027 | 0.263 | 0.246 | *1.578* | 0.139 |
| spinal cord | 0.486 | *1.281* | 0.253 | 0.154 | 0.121 | 0.133 | 0.175 | *2.518* | 0.007 |
| cerebellum | 0.878 | *2.347* | 0.136 | 0.500 | 0.274 | 0.096 | 0.032 | *1.127* | 0.011 |
| fibroblasts | 0.551 | 0.876 | 0.025 | 0.604 | 0.016 | 0.170 | *1.340* | *3.052* | n.d. |

In bold: expression of $>5\%$; in italics: expression of 1–5%; regular letter type: expression of $<1\%$; n.d. no expression of the variant could be detected.

pressed transcripts might represent 'noise', caused by e.g. the relaxation of the RNA splicing machinery. In that case it would be expected that the error rate is similar in all tissues. However, some of the variants show a tissue-specific expression pattern (e.g. NF1-ΔE29, NF1-ΔE45) or are expressed significantly higher in specific tissues (e.g. NF1-insE4a-2, NF1-ΔE37). Our study confirms some of the previous expression data for NF1-ΔE29, NF1-ΔE30 and NF1-ΔE29/30 splice variants in human tissues, but considerably extends those observations by investigating the expression in a larger number of tissues using a more sensitive and quantitative real-time PCR technique. The NF1-ΔE29 transcript, described as being brain-specific [7], was confirmed to exhibit the highest expression in brain and cerebellum. In addition, our study demonstrates a high expression in spinal cord and a low level of expression in other tissues, probably escaping detection on conventional low-sensitivity agarose-based gel electrophoresis [7]. The agreement of our study with a previous one demonstrating a significant difference in variant expression between tissues strengthens the possibility that these transcripts are not the result of noise. Although the NF1-ΔE29, NF1-ΔE29/30 and NF1-ΔE30 variants were reported in a qualitative RT-PCR study [7], we now demonstrate that they account for only a minor proportion of the total *NF1* mRNA. This clearly demonstrates that caution has to be taken for interpretation of non-quantitative PCR end-point results.

Secondly, the variants could represent 'true' functional *NF1* isoforms required in low amounts and might actually have a subtle, yet important biological role in the development of complex organisms. Since the number of genes in humans is roughly only double that of *Caenorhabditis elegans* and alternative splicing probably plays a role in the creation of species diversity, it can be expected that studies in other genes will reveal similar results. Due to the lack of sensitivity of the commonly used quantification techniques (e.g. Northern blotting, RNase protection analysis), the very low expressed variants escape detection. Three recent studies using a similar approach [13–15] also publish data on splice variants (for Jun kinases, 5-$HT_4$ receptors and rat carnitine transporter OCTN2) with expression values of less than 1% of the total transcripts.

A third explanation for the low percentages of alternative *NF1* transcripts might be that expression levels of a specific variant is high in some cells of a specific tissue, but due to pooling of transcripts when preparing RNA from all cells, these transcripts become only a minor fraction of the total. This interpretation is supported by staining patterns of neurofibromin, where it has been shown that the intensity between individual neurons varies dramatically [16,17]. Furthermore, nuclear localization was also seen only in a subset of these neurons and in a subset of NIH3T3 fibroblast cells [16,18]. In cultured keratinocytes a small subpopulation of cells was intensively stained for *NF1* protein, whereas the majority of cells displayed only a weak immunoreaction [19]. Differences in immunoreactivity may reflect differences in physiological state, which could influence the expression of specific *NF1* variants. Microdissection followed by quantification in individual cells might shed more light on this phenomenon.

Two transcripts (NF1-ΔE4b, NF1-ΔE43) displayed high expression in specific tissues. Interestingly, exon 4b represents a mutational hotspot in *NF1*. NF1-ΔE4b results from an out of frame skip of exon 4b and would lead to a premature stop codon. This variant was most highly expressed in blood (5.0%), heart (2.3%) and cerebellum (2.3%), but was also expressed often in several other tissues (Table 2). Skipping of this exon in blood from non-affected people was also described by other groups [12,20] and was shown not to be induced by cold shock. Translation of this variant would lead to a short protein of 163 amino acids lacking the central GAP-related domain involved in the down regulation of RAS [21,22] and hence might be involved in the upregulation of the RAS pathways in different tissues or developmental stages.

Deletion of exon 43 (NF1-ΔE43) is an in-frame event. The NF1-ΔE43 variant had a very high expression in lung, liver, placenta, fetal liver, kidney and skeletal muscle (Fig. 1). Lung, liver and kidney neurofibromin expression is higher during development than in the adult, where expression is barely detectable [16,18]. Skeletal muscle shows a strong expression during development and almost no detectable signal in adults. It is of special interest that NF1-ΔE43 is highly expressed (relative to the general *NF1* expression level) in those tissues where neurofibromin becomes downregulated during late fetal and early postnatal development. We hypothesize that this variant might have a specific function in these tissues. It is

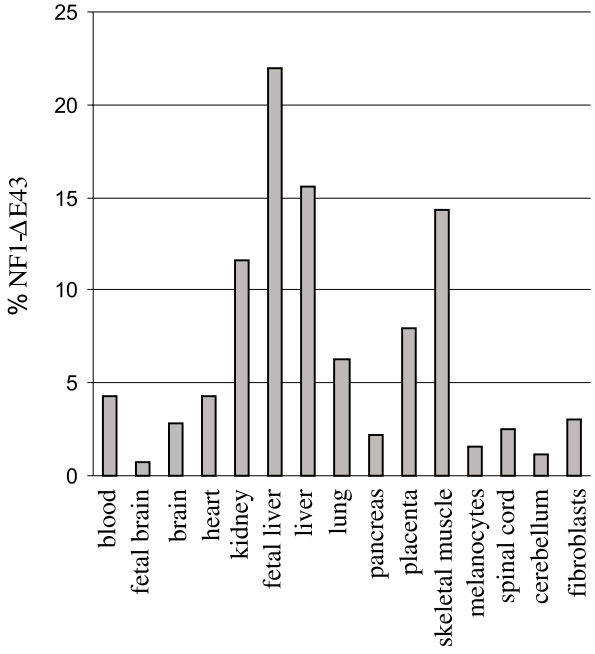


Fig. 1. Expression profiles for NF1-ΔE43 in several human tissues.

noteworthy that these tissues are not typically associated with NF1 pathogenesis. Likewise, the expression of another *NF1* isoform was described as muscle-specific, whereas this tissue is rarely affected in NF1 patients [5,23]. The expression of splice variants in tissues not commonly associated with NF1 pathology raises the question about uncharacterized diseases resulting from defects in the *NF1* gene, but with a different clinical phenotype than NF1. In light of this it is interesting to note that until now no mutation has been found in the alternative exons 9br, 23a and 48a [24–26]. Mutations in exon 43 are also extremely underrepresented with only 2 mutations reported until now [26]. In silico analysis using PROSITE (http://www.expasy.ch/prosite/) revealed that exon 43 contains a bipartite nuclear localization signal. Hence a neurofibromin translated from this transcript might be functionally different from full-length neurofibromin and may be excluded from nuclear localization. Neurofibromin staining in the nucleus has so far been reported for developing neurons [27], in a subset of neurons in adult rat root ganglia [16], in a subset of NIH3T3 fibroblasts [18] and in differentiating keratinocytes [19]. The lower expression of the NF1-ΔE43 transcript in neural tissues versus non-neural tissues might indicate that, if this isoform lacking the nuclear localization signal is formed, nuclear targeting may be important in neural tissues. Up- or downregulation of different *NF1* isoforms could influence the activity and localization of neurofibromin. Antibodies are now being generated against this region in order to characterize this form at the protein level. Further studies will be performed to reveal the localization and function of this variant.

During the finalization of this manuscript an independent study was published also detecting the NF1-ΔE43 variant in fresh blood, in blood using the PAXgene blood RNA tubes, which stabilizes and protects RNA from degradation, and in several tissues, but these analyses were not performed in a quantitative manner [28].

### 3.3. *Quantification of NF1-ΔE43 and NF1-ΔE4b in NF1 patients*

The complex pattern of alternative splicing might provide insights into the extreme phenotypical variability between patients, even among affected members of the same family. The splicing machinery could act as a potential genetic modifier. Interindividual differences in the expression or stability of the different *NF1* variants could influence the phenotypic expression of *NF1* mutations. Differences in the ratio of alternatively spliced transcripts can arise from variations in certain splicing factors or from SNPs situated in splicing enhancers or silencers [29]. We explored this hypothesis by quantification

Table 3
Quantification of NF1-ΔE4b and NF1-ΔE43 in lymphocyte cultures from NF1 patients and healthy control samples

| Type | Mutation | Effect on cDNA | Amino acid change | % NF1-ΔE4b | % NF1-ΔE43 |
|---|---|---|---|---|---|
| Nonsense | 574C > T | 574C > T | R192X | 3.95 | 11.03 |
| Nonsense | 625C > T | 625C > T | Q209X | 2.22 | 11.11 |
| Nonsense | 910C > T | 910C > T | R304X | 1.44 | 14.00 |
| Nonsense[a] | 3826C > T | 3826C > T | R1276X | 1.12 | 3.60 |
| Nonsense[a] | 3826C > T | 3826C > T | R1276X | 1.20 | 11.80 |
| Nonsense | 4084C > T | 4084C > T | R1362X | 1.40 | 4.66 |
| Deletion | 819–821delCCT | 819–821delCCT | frameshift | 2.41 | 8.48 |
| Deletion | 1756–1759delACTA | 1756–1759delACTA | frameshift | 2.71 | 6.01 |
| Deletion | 3457–3460delCTCA | 3457–3460delCTCA | frameshift | 2.31 | 8.39 |
| Deletion | 5717delT | 5717delT | frameshift | 4.87 | 5.06 |
| Large deletion | total gene deletion | – | – | 2.92 | 7.00 |
| Splice | 1261–19G > A | 1261–17ins17 | frameshift | 2.58 | 3.74 |
| Splice | 1466A > G | 1466–1527del62 | Y489C | 1.50 | 3.82 |
| Splice | 1885G > A | 1846–1886del41 | frameshift | 2.93 | 5.87 |
| Splice | 2990+3A > C | 2851–2990del140 | frameshift | 3.43 | 3.27 |
| Missense[b] | 4267A > G | 4267A > G | K1423E | 1.86 | 7.31 |
| Missense[b] | 4267A > G | 4267A > G | K1423E | 1.20 | 5.21 |
| Insertion[c] | 2585insA | 2585insA | frameshift | 3.34 | 4.25 |
| Insertion[c] | 2585insA | 2585insA | frameshift | 2.24 | 5.21 |
| Duplication | dupl23-2 | dupl23-2 | frameshift | 2.42 | 3.05 |
| Mean of 23 control samples | – | – | – | 3.11 | 5.83 |

[a]Non-related patients carrying an identical mutation.
[b]Related patients carrying an identical mutation.
[c]Different lymphocyte cultures from the same patient.

of NF1-ΔE43 and NF1-ΔE4b in RNA freshly extracted from short-term lymphocyte cultures of 19 NF1 patients and 23 control subjects. Patients with different mutation types, including nonsense, frameshift, missense, insertions, deletions and splice site mutations were selected. Two independent lymphocyte cultures, established from the same NF1 patient (2585insA), two cultures from affected members of the same family (4267A > G) and two cultures from non-related NF1-patients carrying an identical mutation (3826C > T) were included. Mean quantities for 23 normal lymphocyte cultures for NF1-ΔE43 and NF1-ΔE4b are 5.8% and 3.1%, respectively (Table 3). These values differ slightly from measurements of RNA extracted immediately from blood and may be attributed to the different multi-cellular composition of lymphocytes isolated directly from blood in comparison with short-term lymphocyte cultures in which selection for T-cells occurs. Variability is observed between different individuals, but comparable results are obtained for the two independent cultures established from the same NF1 patient (Table 3). Markedly, ratios higher than 10% for NF1-ΔE43 all correspond to NF1 patients carrying nonsense mutations. However, a possible correlation was not found using statistical analysis. Moreover, two samples of the control group have similar values (individual data not shown), and two non-related NF1 patients carrying an identical nonsense mutation (3826C > T) exhibit considerably different expression ratios, confirming the lack of correlation. As a conclusion, these quantitative data clearly demonstrate interindividual variations in the expression ratio of NF1-ΔE43 and NF1-ΔE4b.

Mutation analysis of *NF1* gene has shown that about 30% of NF1 patients carry a splice mutation resulting in the production of one or several shortened transcripts [24–26]. Some of the variants quantified in this study are identical to mis-spliced transcripts constitutively formed by specific genomic *NF1* mutations in patients (e.g. NF1-ΔE29, NF1-ΔE29/30 and NF1-ΔE37) [24,25]. Factors modifying the splicing efficiency might also cause variation in the levels of the aberrantly spliced transcripts transcribed from the mutated allele. Disease variability among cystic fibrosis patients carrying splicing mutations has been shown to be associated with varying levels of normally and aberrantly spliced *CFTR* transcripts. In individuals carrying the 5T allele in intron 8 of the *CFTR* gene, which can lead to exon 9 skipping, an extreme variability in the level of exon 9 splicing is observed in conjunction with a variable phenotype in the patients [30]. This has also been described for the 3849+10 kb C→T mutation, leading to an active splice site in intron 19 of the *CFTR* gene, which can lead to the insertion of a 84-bp 'exon'. Variability in the relative amounts of splicing factors probably contributes to the different patterns of alternative splicing found among different individuals and hence to the variation in disease severity [31,32]. It would be interesting to analyze mutated transcripts in *NF1* patients carrying an identical mutation in order to examine this hypothesis.

### 3.4. Demonstration that NF1-ΔE4b, NF1-ΔE37 and NF1-ΔE43 are bona fide transcripts

RNA polymerase II transcripts are subjected to polyadenylation, which has a function in mRNA stabilization, nuclear export and translation. In order to demonstrate that NF1-ΔE4b, NF1-ΔE37 and NF1-ΔE43 are bona fide transcripts, cytoplasmic RNA was extracted from lymphocyte cultures of four healthy individuals and poly($A^+$) mRNA was purified. Real-time PCR quantification results demonstrated the presence of these transcripts (NF1-ΔE4b: 3.7%, NF1-ΔE37: 0.9%, and NF1-ΔE43: 4.3%), confirming that they are polyadenylated and efficiently transported to the cytoplasm.

### 3.5. Determination of conservation of NF1-ΔE4b, NF1-ΔE37 and NF1-ΔE43 variants in mouse

To determine human–mouse conservation of variant expression of NF1-ΔE4b, NF1-ΔE37 and NF1-ΔE43, PCR was performed on mouse cDNA samples (NF1-ΔE4b: heart and lung; NF1-ΔE37: heart and skeletal muscle; NF1-ΔE43: lung and kidney). Primers were designed so that the full-length and skipped transcripts could be amplified simultaneously (Table 1). PCR products were cloned and 200 colonies per tissue were lysed and reamplified with the same primers. Products were separated by agarose gel electrophoresis in order to identify a shorter transcript corresponding to the size of the exon-deleted transcript. None of the clones corresponded to this size.

In addition, real-time PCR using boundary-spanning primers adapted to the mouse *NF1* sequence were used for quantification of mouse NF1-ΔE43 (Table 1). Eight mouse tissues (heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis) were screened for the presence of the NF1-ΔE43 transcript. Real-time PCR resulted in good amplification curves for the full-length transcript, but no signal was obtained after 40 amplification cycles for the transcript lacking exon 43.

It can be concluded that these variants are not conserved in mouse. This is in agreement with previously published discrepancies of *NF1* processing between humans and rodents. Inclusion of exon 23b [33] was found in mouse and rat, but not in humans. The NF1-ΔE29/30 and NF1-ΔE29 was only seen in humans and NF1-ΔE30 was seen in rat, but not in mouse [7]. These variants might account for species-specific differences. A recent study on mouse and human ESTs [34] revealed that only 11% of the detected alternative splice pairs were conserved, suggesting that a substantial fraction of alternative splicing events may be species specific. Alternative splicing is regulated by a complex mechanism involving different trans-acting factors, binding on cis-elements that may be species-, tissue- and developmental stage-specific.

## 4. Conclusion

This study provides a detailed examination of the expression patterns of nine *NF1* splice variants across a variety of human tissues using a quantitative real-time PCR strategy. Several novel splice variants were shown to be highly expressed in specific tissues. These variants might shed more light on possible unknown functional domains and might expand our understanding of the role of neurofibromin. The NF1-ΔE43 variant might play an important role, as it is highly expressed in specific tissues and it misses the nuclear localization signal. Further studies are required to characterize these variants at the protein level, and it will be a challenge to investigate whether quantitative differences between variants might play a role in phenotypic variability among NF1 patients.

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