Human Genetics (2019) 138:73–81
https://doi.org/10.1007/s00439-018-1961-5

ORIGINAL INVESTIGATION

Ultra-deep amplicon sequencing indicates absence of low-grade mosaicism with normal cells in patients with type-1 *NF1* deletions

Anna Summerer[1] · Eleonora Schäfer[1] · Victor-Felix Mautner[2] · Ludwine Messiaen[3] · David N. Cooper[4] · Hildegard Kehrer-Sawatzki[1]

Received: 21 September 2018 / Accepted: 20 November 2018 / Published online: 26 November 2018

Abstract

Different types of large *NF1* deletion are distinguishable by breakpoint location and potentially also by the frequency of mosaicism with normal cells lacking the deletion. However, low-grade mosaicism with fewer than 10% normal cells has not yet been excluded for all *NF1* deletion types since it is impossible to assess by the standard techniques used to identify such deletions, including MLPA and array analysis. Here, we used ultra-deep amplicon sequencing to investigate the presence of normal cells in the blood of 20 patients with type-1 *NF1* deletions lacking mosaicism according to MLPA. The ultra-deep sequencing entailed the screening of 96 amplicons for heterozygous SNVs located within the *NF1* deletion region. DNA samples from three previously identified patients with type-2 *NF1* deletions and low-grade mosaicism with normal cells as determined by FISH or microsatellite marker analysis were used to validate our methodology. In these type-2 *NF1* deletion samples, proportions of 5.3%, 6.6% and 15.0% normal cells, respectively, were detected by ultra-deep amplicon sequencing. However, using this highly sensitive method, none of the 20 patients with type-1 *NF1* deletions included in our analysis exhibited low-grade mosaicism with normal cells in blood, thereby supporting the view that the vast majority of type-1 deletions are germline deletions.

Keywords Neurofibromatosis type-1 · NF1 · *NF1* microdeletions · Nonallelic homologous recombination (NAHR) · Low-grade mosaicism with normal cells · Ultra-deep amplicon sequencing · Next-generation sequencing

Introduction

The most common recurrent mutations in neurofibromatosis type-1 (NF1) are large deletions encompassing the *NF1* gene at 17q11.2 and its flanking regions. These so-called *NF1* microdeletions have been detected in 4.7–11% of all patients with NF1 (MIM #162200) and are associated, in most instances, with a severe clinical phenotype (Cnossen et al. 1997; Rasmussen et al. 1998; Kluwe et al. 2004; Zhang et al. 2015). *NF1* microdeletions are nevertheless rare, occurring with an estimated frequency of 1: 60.000. Different types of large *NF1* deletion are known, which are distinguishable by virtue of their size and the location of their breakpoints. Type-1 *NF1* deletions encompass 1.4-Mb and are characterized by breakpoints located within the low-copy repeats (LCRs) termed NF1-REPa and NF1-REPc. The underlying mutational mechanism of type-1 *NF1* deletions is nonallelic homologous recombination (NAHR) between these LCRs (Dorschner et al. 2000; Jenne et al. 2001; López-Correa et al. 2001). Most type-1 *NF1* deletions exhibit breakpoints located within NAHR hotspots of 4–5 kb, termed paralogous recombination sites 1 and 2 (PRS1 and PRS2) (Forbes et al. 2004; De Raedt et al. 2006; Bengesser et al. 2014; Hillmer et al. 2016, 2017; Summerer et al. 2018). If all *NF1* microdeletions are considered, 70–80% are type-1 which is,

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00439-018-1961-5) contains supplementary material, which is available to authorized users.

✉ Hildegard Kehrer-Sawatzki
hildegard.kehrer-sawatzki@uni-ulm.de

[1] Institute of Human Genetics, University of Ulm, Albert-Einstein-Allee 11, 89081 Ulm, Germany

[2] Department of Neurology, University Hospital Hamburg Eppendorf, 20246 Hamburg, Germany

[3] Department of Genetics, University of Alabama at Birmingham, Birmingham, USA

[4] Institute of Medical Genetics, School of Medicine, Cardiff University, Cardiff CF14 4XN, UK

therefore, the most common type of large *NF1* deletion (Pasmant et al. 2010; Messiaen et al. 2011). Type-2 *NF1* deletions encompass only 1.2-Mb and are mediated by NAHR between the *SUZ12* gene and its pseudogene *SUZ12P* located adjacent to NF1-REPc and NF1-REPa, respectively (Roehl et al. 2010a; Vogt et al. 2012). It has been estimated that type-2 *NF1* deletions account for 10–20% of all large *NF1* deletions (Kehrer-Sawatzki et al. 2004; Messiaen et al. 2011). The third recurrent type of large *NF1* deletion is the so-called type-3 *NF1* deletion, which is characterized by NAHR-mediated breakpoints located within NF1-REPb and NF1-REPc (Bengesser et al. 2010; Pasmant et al. 2010; Zickler et al. 2012). However, type-3 *NF1* deletions are relatively rare, since they comprise only 1.4–4% of all large *NF1* deletions (Pasmant et al. 2010; Messiaen et al. 2011).

NF1 deletions of type-1, 2 and 3 have recurrent breakpoints mediated by NAHR. In contrast, atypical *NF1* deletions exhibit non-recurrent breakpoints and are not mediated by recombination, but instead originate via double strand break repair or replication-associated mechanisms (reviewed by Kehrer-Sawatzki et al. 2017). Atypical *NF1* deletions are heterogeneous in relation to their size and the number of genes located within the deleted region (Vogt et al. 2014 and references therein). Atypical *NF1* deletions account for ~ 10% of all *NF1* microdeletions (Pasmant et al. 2010; Messiaen et al. 2011).

In addition to breakpoint location and underlying mutational mechanism, the various types of *NF1* microdeletion are potentially also distinguishable by the frequency of somatic mosaicism with normal cells not harbouring the *NF1* deletion. Type-2 *NF1* deletions are frequently of postzygotic origin (Kehrer-Sawatzki et al. 2004; Steinmann et al. 2007); indeed, at least 63% of all patients with type-2 *NF1* deletions exhibit somatic mosaicism with normal cells (Vogt et al. 2012). Importantly, type-2 deletions are mostly associated with low-grade mosaicism with normal cells in blood, characterized by low proportions (1–5%) of normal cells as determined by interphase-FISH (Roehl et al. 2012). The number of normal cells in these cases has been noted to be higher in other cell types such as skin fibroblasts and urine-derived cells and hence the detection of low-grade mosaicism with normal cells is somewhat easier in these tissues (Roehl et al. 2012). However, cells other than those derived from blood are often not available for analysis. So far, FISH has been the only method suitable for the detection of low-grade mosaicism.

In addition to type-2 deletions, atypical *NF1* deletions are also frequently of postzygotic origin and it has been estimated that approximately 60% of atypical *NF1* deletions exhibit somatic mosaicism with normal cells (Vogt et al. 2014). However, as yet, the proportion of low-grade mosaicism with normal cells in patients with atypical *NF1* deletions has not been investigated in any detail.

In contrast to type-2 and atypical *NF1* deletions, only a very small proportion (3.4%) of type-1 *NF1* microdeletions have been observed to be of postzygotic origin and associated with somatic mosaicism with normal cells not harbouring the deletion (Messiaen et al. 2011). In the study of Messiaen et al. (2011), four (3.4%) of 116 patients with type-1 *NF1* deletions exhibited mosaicism with normal cells. Only one of these four patients had low-grade mosaicism, with normal cells being present at a level of only 3% in the blood. In one of the four patients, the proportion of normal cells was not determined precisely and the other two patients exhibited 20% and 50% of normal cells in their blood as determined by FISH. Hence, low-grade mosaicism with normal cells would appear to be very rare in patients with type-1 *NF1* deletions, being detected in only 1% of patients with type-1 *NF1* deletions. However, not all 116 patients in the study of Messiaen et al. (2011) have been investigated by FISH. Consequently, some patients with type-1 *NF1* deletions and mosaicism with normal cells might not have been detected, particularly those with a high proportion of cells harbouring the deletion and only low proportions of normal cells (< 10%) in blood.

Low-grade mosaicism with normal cells may have been overlooked in patients with type-1 *NF1* deletions investigated by classical methods used to detect *NF1* deletions such as multiplex ligation-dependent probe amplification (MLPA). This method is characterized by an intrinsic detection limit of 10–20% (Coll-Mulet et al. 2008; Stevens-Kroef et al. 2009; van Veghel-Plandsoen et al. 2011; reviewed by Hömig-Hölzel and Savola 2012; Alhourani et al. 2014). In the case of patients with high proportions of cells with *NF1* microdeletions in their blood, normal cells not harbouring the deletion and present at proportions lower than 10–20% are not detected by MLPA. Similar detection limits are associated with other methods such as microarray analysis and Sanger sequencing (Tsiatis et al. 2010; Davidson et al. 2012; Oneda et al. 2017; Kumar et al. 2018). However, next-generation sequencing methods including ultra-deep amplicon sequencing with high coverage of the target regions (> 1000 sequence reads), have been shown to detect low-grade somatic mosaicism down to a level of only a few percent (reviewed by Cohen et al. 2015; reviewed by Gajecka 2016; Contini et al. 2015; Quin et al. 2016; Bernkopf et al. 2017; Chang et al. 2017; Morimoto et al. 2017).

In the study presented here, we performed ultra-deep amplicon sequencing to investigate the putative presence of low proportions of normal cells in the blood of patients with type-1 *NF1* deletions which were initially identified by MLPA and not associated with somatic mosaicism according to this method. The principle of our approach was to screen for heterozygosity of single nucleotide variants (SNVs) located within the *NF1* microdeletion region which would indicate the presence of normal cells not harbouring the

deletion. To analyse the frequency of somatic mosaicism in patients with type-1 *NF1* deletions is important since it can influence both the phenotypic expression of the disease and the transmission risk (reviewed by Kehrer-Sawatzki and Cooper 2008). Further, although as a group, patients with type-1 *NF1* deletions exhibit a severe clinical phenotype, variability in the clinical manifestations of the disease is frequently observed at the individual level (Mautner et al. 2010). This variation could be associated with somatic mosaicism with normal cells that has remained undetected because it was present as low-grade mosaicism with normal cells in the blood. If low-grade mosaicism with normal cells were very rare in patients with type-1 deletions, the observed inter-individual differences in clinical manifestations might result from other factors such as modifying genes.

Patients and methods

Patients

In this study, genomic DNA derived from blood samples of 20 patients with type-1 *NF1* deletions was analysed by means of ultra-deep amplicon sequencing. The deletion breakpoints of 18 of these 20 patients have been previously determined by means of breakpoint-spanning PCRs (Mautner et al. 2010; Hillmer et al. 2016, 2017; Summerer et al. 2018). The deletions of the remaining two patients were characterized in terms of their breakpoint location during the course of this study. In addition to the 20 patients with type-1 *NF1* deletions, we investigated two patients with either an atypical *NF1* deletion or a type-2 deletion as summarized in Table 1. All 22 patients have been investigated by MLPA (P122 NF1 area probemix, version C2, MRC Holland, The Netherlands) in our previous study and somatic mosaicism with normal cells was not detected by this method (Summerer et al. 2018). The parental origin and the chromosomal mechanism underlying some of these deletions has been determined previously as indicated in Table 1 (Neuhäusler et al. 2018). The clinical features of the patients are summarized in Supp. Table S1. The patients provided written informed consent and the study was approved by the respective institutional review boards.

Ultra-deep amplicon sequencing

In total, 96 amplicons were amplified by PCR using the AmpliTaq Gold™ 360 Master Mix (ThermoFisher Scientific, Waltham, USA) and primers listed in Supp. Table S2. The genomic positions of the amplicons, as well as the SNVs with a minor allele frequency (MAF) ≥ 1% covered by these amplicons, are listed in Supp. Table S3. The amplicons had a mean size of 204-bp (size range 124–266-bp) and were located within the *NF1* microdeletion region as schematically indicated in Fig. 1. In total, 1809 SNVs were covered by these 96 amplicons. Of these 1809 SNVs, 141 SNVs had a MAF ≥ 5% whereas 186 SNVs had a MAF ≥ 1% (Supp. Table S3). The amplicons were purified using Exonuclease I (ThermoFisher Scientific, Waltham, USA) and FastAP thermosensitive alkaline phosphatase (ThermoFisher Scientific, Waltham, USA). All amplicons were quantified by means of the Qubit-4 fluorometer and equal amounts (14 ng) of each amplicon were added to the pool. The amplicons were produced and quantified at the Institute of Human Genetics, University of Ulm. Subsequent experiments were performed by Eurofins Genomics GmbH (Ebersberg, Germany) including the library preparation and adaptor ligation to the amplicons by means of the NEBNext Ultra II DNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA). The indexed libraries were subjected to paired-end sequencing on an Illumina HiSeq 4000 platform performed by Eurofins Genomics GmbH (Ebersberg, Germany). At least five million sequence reads were obtained from each patient-specific library, which were analysed in different time-shifted batches; hence not all libraries were sequenced on the same HiSeq 4000 lane.

The amplicon reads in FastQ file format were aligned against the reference sequence and analysed by means of the SeqNext module of the Sequence Pilot software (JSI medical systems GmbH, Ettenheim, Germany). Filtering of reads was performed according to the default settings: (1) quality score threshold: 10, representing a base call accuracy of at least 90%; (2) ignore reads threshold: 30%, meaning that reads with more 30% of incorrect base calls were not considered. The mean number of reads (coverage) at the sites of SNVs with a MAF ≥ 1% per DNA sample is summarized in Supp. Table S4.

Results

Validation of the ultra-deep amplicon sequencing

In this study, we performed ultra-deep sequencing of 96 amplicons covering a total of 186 SNVs with a MAF ≥ 1% (Supp. Table S3) and investigated the allele frequency and heterozygosity of these SNVs located within the *NF1* microdeletion region (Fig. 1). To evaluate the efficiency of this approach to detect low-grade mosaicism with normal cells, we analysed DNA samples derived from the blood of three patients with mosaic type-2 *NF1* deletions previously identified by either interphase-FISH or microsatellite marker analysis (Roehl et al. 2010a, 2012). Type-2 *NF1* deletions are frequently of postzygotic origin, and patients with these deletions exhibit low numbers (1–10%) of normal cells in blood (Roehl et al. 2012). In the three blood-derived DNA

Table 1 Patients with large *NF1* deletions analysed by ultra-deep amplicon sequencing to investigate low-grade mosaicism with normal cells

Patient	Deletion type	Breakpoint location	Breakpoint[a]	Parental origin[b]		Chromosomal origin[b]		Breakpoint analysis (reference)
				Maternal	Paternal	Inter-chromosomal	Intra-chromosomal	
M-273	Type-1	PRS2	28,994,104–28,994,374		+	n.d	n.d	(3)
2490	Type-1	PRS2	28,995,308–28,995,962		+		+	(3)
1952	Type-1	PRS1	28,976,467–28,976,515		+		+	(2)
4677	Type-1	PRS2	28,995,025–28,995,218	+			+	(5)
SB94	Type-1	PRS2	28,994,104–28,994,374	+			+	(4)
800	Type-1	PRS2	28,994,104–28,994,304	+		+		(3)
1338	Type-1	PRS1	28,974,030–28,974,134	+		n.d	n.d	(3)
2536	Type-1	between PRS1 and PRS2	28,985,786–28,985,945	+		n.d	n.d	(4)
1547	Type-1	PRS2	28,994,985–28,995,225	+		n.d	n.d	(3)
3010	Type-1	PRS2	28,994,722–28,995,010	n.d	n.d	n.d	n.d	(1)
2284	Type-1	PRS2	28,994,656–28,994,985	n.d	n.d	n.d	n.d	(2)
1380	Type-1	PRS2	28,993,242–28,996,294	n.d	n.d	n.d	n.d	(1)
867	Type-1	PRS2	28,994,656–28,994,985	n.d	n.d	n.d	n.d	(2)
521	Type-1	PRS2	28,993,242–28,996,294	n.d	n.d	n.d	n.d	(1)
284	Type-1	PRS2	28,994,656–28,994,985	n.d	n.d	n.d	n.d	(2)
4061	Type-1	PRS1	28,975,239–28,975,390	+	n.d	n.d	n.d	(3)
1778	Type-1	PRS1	28,975,079–28,975,419	n.d	n.d	n.d	n.d	(2)
R002180/1	Type-1	PRS1	28,975,239–28,975,390	n.d	n.d	n.d	n.d	(4)
R610160/101	Type-1	PRS1	28,974,752–28,975,079	n.d	n.d	n.d	n.d	(2)
4631	Type-1	centromeric to PRS1	28,960,263–28,960,416	+		n.d	n.d	(6)
4647	Type-2		n.d	+		n.d	n.d	(7)
R003150/2	Atypical		28,936,803–28,937,073 30,363,856–30,365,250	n.d	n.d	n.d	n.d	(4, 8)

(5) As determined by breakpoint-spanning PCRs during the course of this study; (6) identified by means of array analysis and breakpoint-spanning PCRs in this study; (7) as determined by MLPA in this study; (8) The extent of the atypical deletion in patient R003150/2 has been analysed in our previous study (Summerer et al. 2018). The deletion in this patient was found to be atypical and not mediated by NAHR since the centromeric breakpoint was located within the *SMURF2-P* pseudogene between nucleotide positions 28,936,803 and 28,937,073 (hg19). The telomeric deletion breakpoint was estimated to be located between 30,363,856 and 30,365,250 within the *LRRC37B* gene in NF1-REPc (Summerer et al. 2018)

(1) as determined by Mautner et al. (2010); (2) according to Hillmer et al. (2016); (3) according to Hillmer et al. (2017); (4) according to Summerer et al. (2018);

n.d. not determined

[a]The breakpoints of the type-1 deletions are given as strand exchange regions (SERs) and their genomic position according to NF1-REPa (hg19). The atypical deletion in patient R003150/2 was not mediated by NAHR and the breakpoints in the non-homologous regions of NF1-REPa and NF1-REPc are indicated

[b]As determined by microsatellite marker analysis (Neuhäusler et al. 2018)

samples used to validate our approach, ultra-deep amplicon sequencing detected proportions of normal cells of 5.3%, 6.6% and 15.0%, respectively (Table 2; Supp. Tables S5-S7). The number of heterozygous SNVs per patient was 42, 36 and 45 SNVs, respectively (Supp. Tables S5-S10).

From these validation experiments, we were able to conclude that heterozygosity of SNVs with frequencies of the second allele < 1% were likely to be artefacts caused by sequencing errors (Supp. Tables S8–S10). Further, we deduced from the validation experiments that several SNVs must exhibit heterozygosity for us to be able to conclude that low-grade mosaicism was present. Consanguinity was not reported in relation to the parents of the patients we investigated, who were either of European or North American descent. Hence, autozygosity is unlikely to have impaired our analysis. Further, extended homozygosity of the complete *NF1* deletion region has not been observed by means of SNP array analysis in Europeans (Roehl et al. 2010b). The validation experiments performed here indicated an average of 41 heterozygous SNVs per DNA sample which

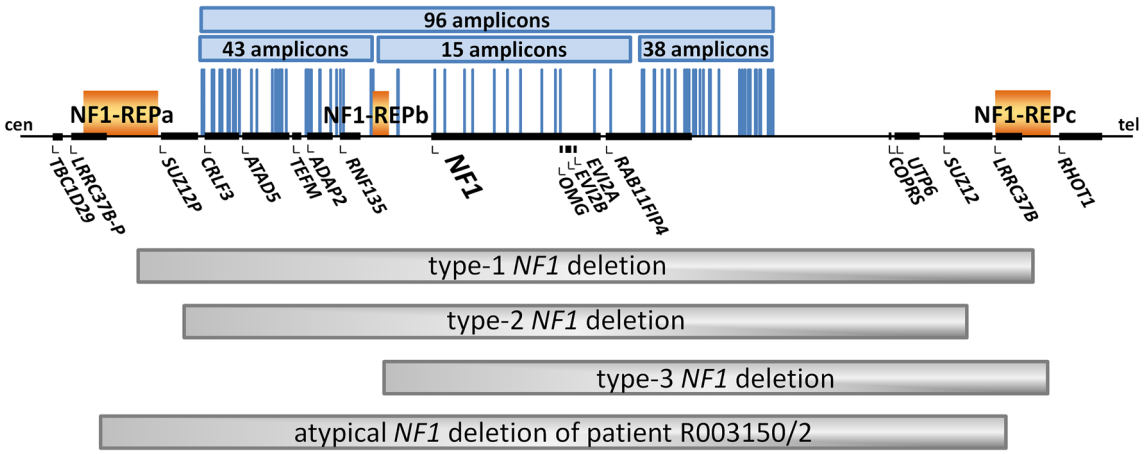

Fig. 1 Schema of the type-1 *NF1* deletion region flanked by the low-copy repeats NF1-REPa and NF1-REPc. Horizontal black bars indicate the genes located in regions flanking the *NF1* gene. The relative positions of the 96 amplicons that have been analysed by ultra-deep sequencing to identify low-grade mosaicism with normal cells are indicated by vertical bars

Table 2 Proportion of normal cells not harbouring the deletion in the blood of patients with type-2 *NF1* deletions

Patient	Number of heterozygous SNVs detected	Proportion of normal cells as estimated by		
		Ultra-deep amplicon sequencing (%)	FISH	Microsatellite marker analysis
1502	42	5.3	4.1%[a]	n.d
1630	36	6.6	3.5%[a]	n.d
UC172	45	15.0	n.d	10–20%[b]

[a]As determined previously by Roehl et al. 2012

[b]Initially, mosaicism was identified in patient UC172 by microsatellite marker analysis of blood-derived-DNA and the identification of heterozygosity for markers located within the deletion region. The second alleles of heterozygous markers located within the deletion region exhibited greatly reduced peak heights as compared to the other alleles of these markers, suggesting that the proportion of cells harbouring the deletion was 80–90% (Roehl et al. 2010b)

demonstrated that the 96 amplicons, designed by us to cover the entire *NF1* deletion interval, are well suited for the reliable detection of low-grade mosaicism with normal cells.

The PCR preceding the ultra-deep sequencing to generate the amplicons could have introduced a quantification bias, both by sequencing errors and by preferential amplification of one allele. It has been shown that nucleotide substitution errors, in particular transitions, introduced during the preceding PCR amplification of the target regions, can impact upon the ultra-deep sequencing results (Brodin et al. 2013; Shao et al. 2013). Site-specific frequencies of substitution errors have been observed in the range of 0.0063–1.17% (Brodin et al. 2013). Additionally, quantification of alleles may be hampered by an amplification bias caused by SNVs at primer binding sites. To reduce the impact of any such bias, we took care to ensure that the three nucleotides at the 3′ end of the PCR primers did not bind to SNVs with a minor allele frequency > 1%. Nevertheless, we cannot exclude the possibility that an amplification bias of certain alleles has influenced our analysis.

To assess this possible quantification bias in greater detail, we analysed the alleles at sites of heterozygous SNVs in patients with mosaic type-2 deletions (Supp. Table S11). The average proportions of the alleles present at lower frequencies and derived from normal cells were 5% in patient 1502, 6.2% in patient 1630 and 13.1% in patient UC172. Our analysis was based on the assumption that the difference between this average and the observed proportion of the less frequently represented allele of the given SNV may reflect a quantification bias. In total, 39 SNVs were informative in the sense that they were heterozygous in at least two patients with mosaic type-2 deletions. For 23 of these 39 SNVs, we observed a bias in favour of the amplification of a specific allele in at least two patients. However, on average this bias amounted only 0.6% of the estimated allele frequency (range 0–3%; Supp. Table S11).

Remarkably, the proportions of normal cells in the blood of the three patients with type-2 deletions were estimated to be higher by ultra-deep amplicon sequencing than by interphase-FISH even though the same blood samples had been analysed (Table 2). This difference may have resulted from the stringent FISH evaluation performed in our previous study which erred on the conservative side by aiming to avoid consideration of false positive normal cells (Roehl et al. 2012). On the other hand, the allele frequencies of heterozygous SNVs determined by ultra-deep sequencing showed some variation when different amplicons derived from the same DNA sample were compared, possibly indicative of a quantification bias as mentioned above (Supp. Figure S1; Supp. Table S11). Nevertheless, our

results clearly indicate that ultra-deep sequencing of the amplicons represents a very sensitive technique for the detection of low-grade mosaicism with normal cells in the blood of patients with high numbers of cells harbouring large *NF1* deletions.

Investigation of potential low-grade mosaicism in 22 patients with large *NF1* deletions

We analysed 22 patients with large *NF1* deletions for the presence of low-grade mosaicism with normal cells. Since interphase-FISH had not been performed with blood samples from any of these 22 patients, low-grade mosaicism with normal cells could not be excluded. Our study cohort included 20 patients with type-1 *NF1* deletions as well as one patient with an atypical *NF1* deletion. The breakpoints of this atypical deletion were found to be located within NF1-REPa and NF1-REPc, but not within regions with high sequence similarity between the LCRs. Thus, the deletion was not mediated by NAHR, as was noted in our previous study (Summerer et al. 2018). Further, we also investigated a patient with a type-2 *NF1* deletion who exhibited a severe clinical phenotype, which is unusual for patients with mosaic type-2 deletions (Table 1, Supp. Table S1).

Eighteen of the 20 patients with type-1 *NF1* deletions had breakpoints located within the NAHR hotspots PRS1 and PRS2 as determined previously (Table 1). Two of these 20 patients had breakpoints located outside these hotspots but within other regions of high sequence homology between NF1-REPa and NF1-REPc. The breakpoints in one of them, patient 4631, had not been previously reported, but were instead determined by microarray analysis and breakpoint-spanning PCR performed during the course of this study. As schematically indicated in Supp. Figure S2, the breakpoints of this NAHR-mediated deletion were found to be located within the *LRRC37B* gene and its pseudogene as determined by the sequence analysis of breakpoint-spanning PCR products.

To investigate the potential presence of low proportions of normal cells in the blood of these 22 patients, we performed ultra-deep sequencing of the 96 amplicons as described above (and validated by means of the confirmed mosaic type-2 *NF1* deletions). In none of the 22 patients was a second allele observed with a frequency > 1% in more than two SNVs (Supp. Tables 12–33). Hence low-grade mosaicism with normal cells was not detected in any of these cases by means of the ultra-deep amplicon sequencing approach.

Discussion

The analysis of low-grade mosaicism with normal cells in patients with large *NF1* deletions is potentially very important in the context of ascertaining genotype/phenotype correlations. So far, FISH has been the method of choice for the investigation of low-grade mosaicism but this method has its application limits since it is dependent upon the availability of (1) fresh blood samples, (2) high quality FISH probes which are not commercially available and (3) experienced cytogeneticists to assess low-grade mosaicism with normal cells to a high degree of accuracy. The study presented here is the first to demonstrate that ultra-deep amplicon sequencing is well suited to detect low-grade mosaicism of normal cells in NF1 patients with high numbers of cells harbouring large *NF1* deletions. Our validation experiments using three mosaic type-2 *NF1* deletions previously identified by interphase-FISH or microsatellite marker analysis indicated low proportions (5.3%, 6.6% and 15.0%) of normal cells in the blood of the patients (Supp. Tables S5-S7; Table 2). Type-2 *NF1* deletions are frequently of postzygotic origin associated with high numbers of cells harbouring the deletion in blood (Kehrer-Sawatzki et al. 2004; Vogt et al. 2012). In contrast, the frequency of low-grade mosaicism with normal cells in patients with other types of large *NF1* deletions has not as yet been investigated in any detail. Previous studies have suggested that only a small proportion of type-1 *NF1* microdeletions are of postzygotic origin associated with somatic mosaicism with normal cells (Messiaen et al. 2011). In the latter study, low-grade mosaicism characterized by 3% of normal cells was detected in only one of 116 patients with type-1 *NF1* deletions investigated. However, not all 116 patients in the study of Messiaen et al. (2011) have been analysed by FISH. Consequently, some patients with type-1 *NF1* deletions and mosaicism with normal cells may not have been detected, particularly those with low proportions of normal cells (< 10%) in blood.

To further investigate the frequency of low-grade mosaicism with normal cells in patients with type-1 *NF1* deletions, we performed ultra-deep amplicon sequencing in 20 patients with type-1 *NF1* deletions, which is as yet the most sensitive method to detect low-grade mosaicism. However, proportions of second alleles > 1% of more than two SNVs located within the *NF1* deletion region, indicative of the presence of normal cells, were not detected (Supp. Tables S12-S33). This finding implies that low-grade mosaicism with normal cells is indeed very rare in patients with type-1 *NF1* deletions. Nevertheless, since cells other than blood-derived cells were not available to us for analysis, the presence of low-grade mosaicism in the patients with type-1 *NF1* deletions investigated cannot be completely excluded. As mentioned above, patients with mosaic type-2 *NF1* deletions frequently exhibit high numbers (> 90%) of cells harbouring the deletion in blood but lower proportions of cells with the deletion in other tissues (Roehl et al. 2012). We analysed three type-2 *NF1* deletions exhibiting high proportions of cells with the deletion in blood (94.6%, 93.4% and 85.0%) by means of ultra-deep sequencing and mosaicism with normal

cells was readily detected. Hence, we believe that ultra-deep sequencing is indeed a powerful technique to detect low-grade mosaicism with normal cells, even if the proportion of cells harbouring the deletion is very high. Our analyses of 20 patients with type-1 deletions as well as the validation experiments we performed, imply that the detection limit of the ultra-deep sequencing approach is around 1%. In other words, normal cells present in a proportion < 1% would have gone undetected. Our findings indicate that at least 99% of cells in the blood of the type-1 deletions investigated by us harboured the deletion and if normal cells were present, their proportion would have been lower than 1%.

The assumption that the majority of type-1 *NF1* deletions are of meiotic origin and hence not associated with somatic mosaicism is supported by the observation of a pronounced maternal parent-of-origin bias for type-1 *NF1* deletions and the predominance of interchromosomal NAHR causing these deletions. Previous analyses have shown that ~91% of all type-1 *NF1* deletions are of maternal origin and predominantly caused by interchromosomal NAHR (observed in 87.5% informative cases) (López-Correa et al. 2000; Neuhäusler et al. 2018). Interchromosomal exchange of maternal chromosomes causing type-1 deletions most likely occurs by unequal crossover during meiosis I, when the homologous chromosomes are joined by the synaptonemal complex thereby bringing them into close proximity to each other for an extended period of time. By contrast, type-2 *NF1* deletions, which are frequently of postzygotic origin, are mediated by intrachromosomal NAHR and any preference for a parental origin has not been observed (Roehl et al. 2010a).

It may be possible that some of the less frequent type-1 *NF1* deletions of paternal origin and those mediated by intrachromosomal NAHR could be of postzygotic origin, in contrast to the *NF1* deletions mediated by interchromosomal NAHR which are more likely to occur during meiosis. These deletions are, however, rare and family analysis including siblings by means of microsatellite marker analysis is necessary to identify the parental and chromosomal origin of the deletions. In the study presented here, we analysed three paternal type-1 *NF1* deletions as well as two maternally derived type-1 deletions mediated by intrachromosomal NAHR (Table 1). In total, we analysed five of the rare type-1 deletions mediated by intrachromosomal NAHR. Even in these rare forms of type-1 *NF1* deletion, low-grade mosaicism with normal cells was not detected by ultra-deep amplicon sequencing which is the most sensitive method available to detect this type of mosaicism.

We conclude that low-grade mosaicism with normal cells, frequently observed in patients with type-2 *NF1* deletions, is very rare in patients with type-1 deletions. Type-1 deletions seem to occur in most instances in the germline of a parent and are then transmitted to the child. Ectopic recombination occurring during meiosis between misaligned chromosomes 17 predominantly occurs within the NAHR hotspots located in NF1-REPa and NF1-REPc and not within the paralogous *SUZ12* gene and its pseudogene adjacent to these LCRs. By contrast, *SUZ12* and *SUZ12P* harbour the breakpoints that result from mitotic NAHR which must occur early during embryonic development, giving rise to mosaic type-2 deletions (Roehl et al. 2012). One explanation for this could be that the SUZ12 protein is an essential part of the polycomb repressive complex 2 (PRC2) which is required for embryonic stem cell differentiation and development (Bracken et al. 2006; Pasini et al. 2007). Hence transcription of *SUZ12* increases during early embryonic development which may result in an open chromatin conformation that is vulnerable to DNA double strand break formation and transcription-associated recombination (reviewed by Gottipati and Helleday 2009, reviewed by Marnef et al. 2017). Nevertheless, the reasons for these positional preferences of meiotic vs. mitotic recombination breakpoints are still unclear and further studies will be necessary to investigate whether structural genomic variants, such as large inversions of the *NF1* gene region, might exist that could predispose to misalignments of chromosomes 17 during meiosis and NAHR-mediated breakpoints within NF1-REPa and NF1-REPc causing germline type-1 *NF1* deletions.

Acknowledgements This work has been funded by the Deutsche Forschungsgemeinschaft (DFG) grant KE 724/12-2.

Compliance with ethical standards

Conflict of interest The authors are unaware of any conflict of interest.

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