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Tissue-Specific Differences in the Proportion of Mosaic Large *NF1* Deletions are Suggestive of a Selective Growth Advantage of Hematopoietic del(+/–) Stem Cells

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ABSTRACT: Type-2 *NF1* deletions spanning 1.2 Mb are frequently of postzygotic origin and hence tend to be associated with mosaicism for normal cells and those harboring the deletion (del(+/–) cells). Eleven patients with mosaic type-2 deletions were investigated by FISH and high proportions (94–99%) of del(+/–) cells were detected both in whole blood and in isolated CD3+, CD14+, CD15+, and CD19+ leukocytes. Significantly lower proportions of del(+/–) cells (24-82%) were however noted in urine-derived epithelial cells. A patient harboring an atypical large *NF1* deletion with nonrecurrent breakpoints was also found to have a much higher proportion of del(+/–) cells in blood (96%) than in urine (51%). The tissue-specific differences in the proportions of del(+/–) cells as well as the X chromosome inactivation (XCI) patterns observed in these mosaic patients suggest that the majority of the deletions had occurred before or during the preimplantation blastocyst stage before the onset of XCI. We postulate that hematopoietic del(+/–) stem cells present at an early developmental stage are characterized by a selective growth advantage over normal cells lacking the deletion, leading to a high proportion of del(+/–) cells in peripheral blood from the affected patients.

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KEY WORDS: *NF1*; neurofibromatosis type 1; microdeletion; mosaicism; mitotic NAHR

Introduction

Large deletions of the entire *NF1* gene and its flanking regions are present in approximately 5% of all patients with neurofibromatosis type 1 (NF1; MIM# 613113) [Kluwe et al., 2004]. Four distinct types of *NF1* deletion (type-1, type-2, type-3, and atypical) have been identified that are distinguishable in terms of the extent of the deleted region and the location of their respective breakpoints. Type-1 *NF1* deletions encompass 1.4 Mb (including 14 genes) with breakpoints that are located within low-copy repeats, termed NF1-REPa and NF1-REPc [Dorschner et al., 2000; Jenne et al., 2001; López-Correa et al., 2001]. Type-1 deletions are detected in 70–80% of all patients with large *NF1* deletions and hence represent the most frequent *NF1* deletion type [Messiaen et al., 2011; Pasmant et al., 2010]. By contrast, type-2 deletions occur much less frequently, their estimated frequency being 9–20% of all large *NF1* deletions [Kehrer-Sawatzki et al., 2004; Messiaen et al., 2011; Pasmant et al., 2010]. Type-2 *NF1* deletions are also smaller, spanning 1.2 Mb and including only 13 genes. The breakpoints of type-2 deletions are located within *SUZ12* and its pseudogene *SUZ12P* [Kehrer-Sawatzki et al., 2004; Petek et al., 2003; Roehl et al., 2010; Steinmann et al., 2007]. A third type of recurrent *NF1* deletion (type-3) has been reported recently [Bengesser et al., 2010; Pasmant et al., 2010; Zickler et al., 2011]. Type-3 deletions encompass 1.0 Mb and include 9 genes, with breakpoints located within the *LRRC37B*-P paralogs of NF1-REPb and NF1-REPc. Type-3 *NF1* deletions are rare, being observed in only 1–4% of patients with *NF1* deletions [Messiaen et al., 2011; Pasmant et al., 2010]. In addition to these three types of recurrent *NF1* deletion, mediated in most instances by nonallelic homologous recombination (NAHR), a number of atypical *NF1* deletions have also been identified with nonrecurrent breakpoints [Kehrer-Sawatzki et al., 2003, 2005, 2008; Mantripragada et al., 2006; Messiaen et al., 2011; Pasmant et al., 2009, 2010]. By definition, atypical *NF1* deletions are heterogeneous in terms of their size, breakpoint position, and the number of genes included within the deleted region.

Mosaicism with normal cells is an important issue to address in NF1 patients with large *NF1* deletions because it can influence both the phenotypic expression of the disease and the transmission risk [Erickson, 2010; Gottlieb et al., 2001; Kehrer-Sawatzki and Cooper, 2008; Youssoufian and Pyeritz, 2002]. It has been estimated that mosaicism with normal cells occurs in at least 10% of patients with *NF1* microdeletions [Messiaen et al., 2011]. However, this may well represent an underestimate of the true prevalence of mosaic *NF1* deletions because the parents of apparently sporadic patients with deletions are generally not systematically analyzed in terms of whether they exhibit mosaicism for the *NF1* deletion detected in their offspring.

Additional Supporting Information may be found in the online version of this article.

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This problem may not be relevant in the case of the most frequent type of *NF1* deletion (type-1), which appears to be only rarely associated with mosaicism; an estimated 2–4% of all patients with de novo type-1 *NF1* deletions are mosaic deletions [Messiaen et al., 2011]. By contrast, type-2 deletions frequently arise during postzygotic cell division giving rise to somatic mosaicism with normal cells coexisting alongside those harboring the deletion [Kehrer-Sawatzki et al., 2004; Petek et al., 2003; Roehl et al., 2010; Steinmann et al., 2007]. At least 44% of all patients with type-2 *NF1* deletions exhibit somatic mosaicism [Messiaen et al., 2011]. However, in practice, the proportion of patients with mosaic type-2 *NF1* deletions may well be considerably higher [Kehrer-Sawatzki et al., 2004]. In our previous studies, we observed that the proportions of cells harboring the deletion in blood samples of patients with type-2 deletions are very high, in most cases exceeding 90% [Kehrer-Sawatzki et al., 2004, Roehl et al., 2010; Steinmann et al., 2007]. Hence, FISH analysis is necessary to detect mosaicism with normal cells in cases of type-2 *NF1* deletions because low rates of normal cells are not detectable either by array comparative genomic hybridization or PCR-based mutation analysis techniques such as multiplex ligation-dependent probe amplification (MLPA).

Here, we have investigated whether leukocyte subtypes and urine-derived epithelial cells from patients with mosaic *NF1* deletions exhibit differences in the proportion of cells harboring the deletion as compared with whole blood. Eleven patients with type-2 *NF1* deletions were investigated alongside two patients with mosaic *NF1* deletions other than type-2, in order to determine whether high proportions of cells harboring the deletion in blood might be characteristic of the type-2 deletion. Additionally, we determined the X chromosome inactivation (XCI) pattern in female patients with mosaic *NF1* deletions in blood, buccal epithelium, or urine in an attempt to ascertain the timing of deletion formation during embryonic development.

Patients and Methods

Patients and Cell Lines

The proportion of cells harboring the deletion (del(+/–) cells) was analyzed by FISH and microsatellite markers in 11 patients with mosaic type-2 *NF1* deletions. The precise locations of the deletion breakpoints of 10 of these 11 patients were determined in our previous study [Roehl et al., 2010; Steinmann et al., 2007], whereas the breakpoints of the newly identified deletion of patient 2442 were characterized in this study using previously described methods. The nucleotide positions of the deletion breakpoints of all 11 patients with type-2 *NF1* deletions are listed in Supp. Table S1. Mosaicism was also investigated in patients 3304 and 1860-M with *NF1* deletions other than type-2. The breakpoints of the atypical deletion in patient 1860-M were previously identified [Steinmann et al., 2008] whereas the extent of the atypical or type-1 *NF1* deletion of patient 3304 has been characterized in this study using FISH (Supp. Fig. S1) and breakpoint-spanning PCR employing the primers listed in Supp. Table S2. The clinical phenotypes of all three patients newly described in this study are given in Supp. Table S3.

We assessed the proportion of del(+/–) cells in all 13 patients harboring mosaic *NF1* deletions. FISH analysis was performed on cells from whole blood cultured for 72 hr in the presence of phytohemagglutinin and on whole blood cells that were directly prepared for FISH analysis without prior culture. We also used FISH to investigate various leukocyte subtypes isolated from whole blood, which were positive for specific cell surface antigens (CD3+, CD19+, CD14+, and CD15+), and urine-derived epithelial cells. In one of the 11 patients with mosaic type-2 deletions (patient 488), we also used FISH to determine the proportions of del(+/–) cells in fibroblast cultures from skin overlying three different cutaneous neurofibromas as well as primary cells from these tumors.

In order to determine the error rate of the FISH procedure employed, blood samples of six patients with non-mosaic (germline) *NF1* deletions were investigated (listed in Supp. Table S4). Two of these six patients had an inherited atypical deletion (patients 1860 and 1860-1) whereas four patients had sporadic *NF1* deletions. The deletion in patient 1860 spans 1.3 Mb and is atypical [Steinmann et al., 2008; Supp. Fig. S2]. Patient 1860 inherited the deletion from her mother (1860-M) who exhibits mosaicism for the deletion. Patient 1860 passed on the atypical *NF1* deletion to her son, patient 1860-1 (Supp. Fig. S3).

The control group of non-mosaic *NF1* deletion patients included two with a type-1 deletion (patients 3175 and 3415), patient 2358 with a non-mosaic type-2 deletion and patient BUD with a germline atypical *NF1* deletion spanning 4.7 Mb as previously reported [Kehrer-Sawatzki et al., 2003]. The error rate intrinsic to the FISH procedure was assessed in blood and isolated leukocyte subtypes of these patients as summarized in Supp. Table S4. Additionally, we investigated the intrinsic error rate of the FISH analysis using EBV-transformed lymphoblastoid cell lines from a patient with a non-mosaic (germline) type-2 *NF1* deletion (patient 2429), previously characterized by Roehl et al. [2010], and two patients with type-1 *NF1* deletions (patients 1746 and 2490.1). Both these patients had type-1 deletions with breakpoints located within the paralogous recombination site 2 (PRS2) hotspot as determined by breakpoint-spanning PCR [Mautner et al., 2010].

Separation of Leukocyte Subtypes from Whole Blood

In order to isolate different leukocyte subtypes from whole blood samples, we performed magnetic-activated cell separation (MACS, Myltenyi Biotec, Bergisch Gladbach, Germany). For the separation of each of the respective leukocyte subtype, 4-ml whole blood were incubated for 15 min at 4°C with antibodies binding to specific cell surface epitopes and conjugated to ferromagnetic beads. These antibodies were used to isolate CD3-positive cells (T-lymphocytes, T-helper cells, and cytotoxic T-cells), CD19+ B-lymphocytes, CD14+ monocytes, and CD15+ granulocytes, respectively. Subsequently, cells were washed with separation buffer (autoMACS Running Buffer). After centrifugation for 10 min at room temperature, the isolated cells were resuspended in 3-ml separation buffer. Immunomagnetic cell separation was performed with the whole blood column kit. Finally, the bead-isolated cells were eluted in 5-ml elution buffer and centrifuged again. Cytogenetic slides were prepared after incubation of the cells in hypotonic solution (0.4% KCl) and fixation with methanol and acetic acid (3+1).

The efficiency of the separation procedure was assessed initially by the analysis of a blood sample from a healthy individual. In this control experiment, leukocyte subtypes were isolated as described above. Subsequently, the bead-isolated cells were incubated with specific fluorescence-labeled antibodies in order to determine the number of cells positive for the respective epitope and hence the isolation efficiency (which ranged from 97.7–99.0%; Supp. Table S5).

Skin Fibroblasts and Neurofibroma-Derived Cells

Three cutaneous neurofibromas were excised from different skin areas (trunk, head, and neck) of patient 488 and immediately transferred into Dulbecco's modified Eagle's medium (DMEM) with

10% fetal calf serum (FCS) and 0.01% gentamicin. The skin overlying the three neurofibromas was removed and cut into smaller pieces, which were transferred into cell culture flasks to establish skin fibroblast cultures and then grown in DMEM with 10% FCS. After 51 days of cell culture, the fibroblasts were detached from the cell culture vessel surface by trypsin treatment, incubated in hypotonic solution (0.4% KCl) for 30 min and transferred onto slides after fixation to perform FISH analysis.

Cells were isolated from neurofibromas by cutting the tumors into small pieces followed by digestion of these pieces in DMEM with 10% FCS and collagenase type-1 (160U/ml, Biochrom, Berlin, Germany) at 37°C overnight. The following day, the tumor pieces were completely dissolved using a pipette. After centrifugation, the cells were incubated in hypotonic solution (0.4% KCl) for 30 min, centrifuged again, and fixed with a mixture of methanol and acetic acid (3+1). Finally, the cells were transferred onto slides for FISH analysis.

Fluorescence in Situ Hybridisation (FISH)

Slides were prepared for interphase FISH from uncultured whole blood samples, blood samples cultured for 72 hr in presence of phytohemagglutinin, bead-isolated leukocyte subtypes and epithelial cells from urine samples. At least 100-ml urine was necessary to obtain slides containing sufficient numbers of epithelial cells. FISH was also performed on cultured skin fibroblasts and neurofibroma-derived primary cells from patient 488. Dual-color FISH was performed with BAC RP11-142O6, which spans the proximal part of the *NF1* gene (Supp. Fig. S1), and the alpha-satellite enumeration probe SE17/D17Z1 (Kreatech, Amsterdam, Netherlands), as previously described [Jenne et al., 2003]. The extent of the *NF1* deletion in patient 3304 has not been previously reported and was determined in this study by FISH using BAC and PAC clones (whose relative genomic location is schematically indicated in Supp. Fig. S1).

Since FISH is inherently error prone and highly dependent upon both the quality of the FISH probe and the slides used for the analysis, we took great care to optimize hybridization quality and only analyzed those FISH slides with fewer than 5% of nuclei that were difficult to evaluate in terms of the number of signals. Thus, FISH slides with >5% of cells that were difficult to evaluate were excluded from the analysis. All slides were investigated by two persons who performed their analyses independently from one another. The final results represented the average of these two independent evaluations.

Estimation of the Error Rate of the FISH Technique

The number of del(+/–) cells has been found to be very high in blood samples of patients with mosaic type-2 *NF1* deletions, as determined by FISH. To estimate the intrinsic error rate of the FISH analysis, in particular the rate of falsely detected normal cells with two signals pertaining to the *NF1* probe RP11-146O2, we performed FISH on whole blood cells, bead-selected leukocyte subtypes, and urine-derived cells from patients with non-mosaic *NF1* deletions (Supp. Tables S4, S6, and S7). For this purpose, we also investigated EBV-transformed lymphoblastoid cells from two patients with type-1 *NF1* deletions and one patient with a non-mosaic type-2 *NF1* deletion (Supp. Table S8).

Breakpoint-Spanning PCRs

The patients with type-1 *NF1* deletions, who were included in this study as controls in order to determine the error rate of the FISH procedure, were analyzed by breakpoint-spanning PCRs. These PCRs were designed to detect breakpoints located in the meiotic NAHR hotspots PRS1 and PRS2 as well as in the breakpoint region 160P1-F/ MD1-R identified by Forbes et al. [2004] in a single patient with a type-1 *NF1* deletion (Supp. Table S2).

Microsatellite Marker Analysis

DNA was extracted from blood and urine samples using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and from buccal swabs with the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany). Primers used for the analysis of microsatellite markers located within the *NF1* microdeletion region are listed in Supp. Table S9.

Statistical Analysis of the Proportion of del(+/–) Cells in Pair-Wise Comparisons of Leukocyte Subtypes

In order to investigate whether the proportion of del(+/–) cells was significantly higher in CD14+ cells than in other leukocyte subtypes or as detected in whole blood, we performed pair-wise comparisons of the proportions of del(+/–) cells in each of the 11 patients with mosaic type-2 *NF1* deletions. These comparisons were performed between the proportion of del(+/–) cells in CD14+ cells and the proportions detected in CD3+, CD15+, CD19+ cells or whole blood, respectively. The statistical significance of the observed differences was assessed using the Wilcoxon signed rank test.

X Chromosome Inactivation (XCI)

To investigate XCI, we analyzed the polymorphic CAG repeat in exon 1 of the androgen receptor (*AR*) gene located at Xq12 by means of the human androgen receptor (*HUMARA*) methylation-based assay as described by Allen et al. [1992]. DNA methylation of this region within the *AR* gene is highly correlated with X-inactivation status [Allen et al., 1992]. In brief, heterozygosity for the repeat was determined by PCR with primers ARP3 5′-TCC AGA ATC TGT TCC AGA GC-3′ labelled with 6-FAM and ARP4 5′-CTC ATC CAG GAC CAG GTA GC-3′. The PCR products were separated and analyzed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA). Nine of the 11 female patients with mosaic *NF1* deletions investigated were found to be heterozygous for the CAG repeat within the *AR* gene and hence were informative. The methylation-based PCR test was performed by restriction digestion of 1-μg genomic DNA with the methylation-sensitive restriction enzyme *Hpa*II for 2 hr. Complete restriction of the genomic DNA was investigated by control experiments using plasmid DNA (pUC18). For this purpose, 100-ng plasmid DNA was added to an aliquot of the restriction assay with genomic DNA of the respective patient. After a 2-hr incubation, the plasmid control restriction assay was analyzed by agarose gel electrophoresis; the distinctive bands of the plasmid DNA were held to denote complete restriction. Subsequently, PCR was performed, with the abovementioned primers being used to amplify the *AR* repeat, using the *Hpa*II-restricted genomic DNA of the given patient as a template. The resulting PCR products were separated by capillary electrophoresis on an ABI Prism 3100 Genetic Analyzer. The *AR* alleles were analyzed using Genescan and the area under the curve (AUC) of each allele was measured with the Genotyper software in order to calculate the ratio of the active/inactive X chromosome (XCI ratio). The *HUMARA* assays were performed two to four times for each patient or tissue. Finally, the mean AUC value for each allele was determined in order to assess the proportion of cells harboring the higher molecular weight allele A2 on the active X chromosome as described by Swierczek et al. [2008] and

Table 1. Proportions of Cells Harboring the Deletion as Determined by FISH Analysis of Whole Blood Samples and Leukocyte Subtypes from Patients with Mosaic *NF1* Deletions

		Proportion (%) of cells with the deletion in						
		Whole blood		Leukocytes positive for				
Patient	*NF1* deletion	Uncultured[a]	Cultured[b]	CD3	CD19	CD14	CD15	Urine
697	Type-2	98.7	95.9	95.7	95.4	97.7	98.1	27.6
736	Type-2	96.8	96.3	93.8	98.7	99.6	98.8	26.7
488	Type-2	99.0	96.8	99.6	99.5	100	99.0	46.5
1502	Type-2	95.9	97.3	97.3	99.3	99.5	99.3	54.6
928	Type-2	98.7	98.9	97.2	99.6	99.8	99.3	61.3
938	Type-2	97.9	93.5	95.1	96.8	99.6	99.5	23.9
2442	Type-2	99.3	98.6	99.4	98.6	100	99.5	51.7
1956	Type-2	97.5	97.9	96.7	98.8	99.1	97.9	60.0
UC172	Type-2	93.8	94.4	91.9	97.9	94.2	97.8	64.5
585	Type-2	94.9	96.7	94.5	94.3	96.4	94.5	81.9
1630	Type-2	96.5	94.2	94.1	95.1	98.2	97.3	29.3
3304[c]	Atypical or type-1	16.9	18.1	17.6	17.9	17.5	19.3	25.0
1860-M[d]	Atypical	95.7	95.9	94.5	95.1	94.4	94.5	50.9

[a] Whole blood was directly prepared for FISH analysis without prior culturing of blood cells.
[b] Blood samples were cultured for 72 hr with phytohemagglutinin that stimulates the proliferation of T-lymphocytes.
[c] Patient 3304 has a large *NF1* deletion (either atypical or type-1) as determined by FISH (Supp. Fig. S1).
[d] Patient 1860-M has an atypical *NF1* deletion of 1.3 Mb as previously described [Steinmann et al., 2008].

calculated by means of the formula given below. This formula includes a correction for the preferential PCR amplification of the allele (A1) with the lower molecular weight. To perform this correction, the fraction of *Hpa*II-digested *AR* alleles (A1 and A2) was divided by the fraction of the undigested *AR* alleles (A′1 and A′2).

$$\%A2_{active} = 1 - \left\{ \frac{\frac{A2/(A2+A1)}{A'2/(A'2+A'1)}}{\frac{A2/(A2+A1)}{A'2/(A'2+A'1)} + \frac{A1/(A2+A1)}{A'1/(A'2+A'1)}} \right\} \times 100$$

By definition, skewed XCI was considered if ≥75% of the cells inactivate the same X- chromosome as employed in several previous studies [e.g., Bolduc et al., 2008; Busque et al., 2006].

Results

Error Rate of the FISH Analysis

FISH is quite adequate to the task of analyzing mosaicism in patients with large *NF1* deletions because single cells can be analyzed. However, FISH is inherently error prone and hence false-positive signals may occur. For example, low rates of normal cells may be difficult to assess with any degree of precision owing to incorrectly identified "normal" cells with two signals from the *NF1* FISH probe. To assess the error rate inherent to the FISH assay as performed here (i.e., the rate of false-positive normal cells), we analyzed whole blood samples and isolated leukocyte subtypes of six patients with non-mosaic (germline) *NF1* deletions (who would naturally be expected to harbor the deletion in 100% of their cells). The investigated non-mosaic *NF1* deletions were of three types: atypical, type-1, and type-2. The error rate of false-positive normal cells was on average 0.2% and never exceeded 0.8% (Supp. Tables S4 and S6). A low error rate of false-positive normal cells (0.2–0.3%) was also detected by FISH of EBV-transformed lymphoblastoid cells from three patients with non-mosaic *NF1* deletions (Supp. Table S8). We conclude that the error rate of the FISH procedure as performed in this study is low and has not confounded our analysis.

Table 2. Pair-Wise Comparison of the Proportion of del(+/–) Cells in CD14+ Cell Fractions with those Detected in other Leukocyte Subtypes or in Whole Blood

Pair-wise comparisons of the proportion of del(+/–) cells in	Median difference of the proportions of del(+/–) cells (range of the differences in %)	*P*-value
CD14+ cells and uncultured blood	1.5% (–1.0, 3.6)	0.006
CD14+ cells and cultured blood	1.8% (–0.3, 6.1)	0.005
CD14+ cells and CD3+ cells	2.3% (0.4, 5.8)	0.001
CD14+ cells and CD19+ cells	0.9% (–3.7, 3.1)	0.051
CD14+ cells and CD15+ cells	0.5% (–3.6, 1.9)	0.096

The median differences as well as the range of differences between these proportions were determined using the Wilcoxon signed rank test.
These pair-wise comparisons indicated the difference between these proportions and were determined for each of the 11 patients with mosaic type-2 *NF1* deletions. From these data, the median difference was determined.

FISH Analysis of Leukocyte Subtypes from Patients with Mosaic *NF1* Deletions

In blood samples of patients with mosaic type-2 *NF1* deletions, we have previously detected very high proportions (>91%) of cells harboring the deletion (del(+/–) cells) [Kehrer-Sawatzki et al., 2004; Roehl et al., 2010; Steinmann et al., 2007] (Supp. Table S10). In the present study, we investigated whether differences might exist in terms of the proportion of del(+/–) cells in leukocyte subtypes (CD3+, CD19+, CD14+, and CD15+) from the 11 patients with mosaic type-2 *NF1* deletions. FISH analysis did not, however, reveal any specific leukocyte subtype that showed a consistently higher proportion of normal cells as compared with whole blood (Table 1; Supp. Table S11). Remarkably, the proportions of del(+/–) cells detected in CD14+ monocytes were significantly higher than in CD3+ cells, CD19+ lymphocytes, or in whole blood samples (Table 2). However, the difference between the proportions of del(+/–) cells observed in CD14+ monocytes and CD15+ granulocytes was not significant ($P = 0.096$; Table 2, Supp. Table S11).

We also investigated whether the proportion of del(+/–) cells differed between blood samples taken from the same patient at different time points. We compared the proportions of del(+/–) cells

in cultured blood from the nine type-2 deletion patients analyzed here with the proportions determined by us several years ago using the same methodology [Roehl et al., 2010; Steinmann et al., 2007]. In seven of the nine patients, the proportions of del(+/–) cells determined at two different time points were very similar, with the differences between the proportions not exceeding 3%. In two out of nine patients, however, the blood samples taken at these different times exhibited differences in the proportions of del(+/–) cells of 6% and 8%, respectively (Supp. Table S10).

In the present study, we also investigated mosaicism in the leukocyte subtypes of two patients with atypical or type-1 *NF1* deletions (patients 3304 and 1860-M). The extent of the deletion in patient 3304 was investigated by FISH and breakpoint-spanning PCRs. These PCR assays were designed to detect breakpoints located in the NAHR hotspots PRS1 and PRS2 that contain the breakpoints of 89% of all type-1 *NF1* deletions [De Raedt et al., 2006]. Further, we investigated whether patient 3304 was positive for the deletion breakpoint-spanning PCR that detected the breakpoint in patient UWA-160 corresponding to a type-1 deletion, as described by Forbes et al. [2004]. Patient 3304 was, however, negative for these breakpoint-spanning PCRs, and hence the presence of a type-1 *NF1* deletion with breakpoints in these regions could be excluded. FISH analysis of patient 3304 indicated that his deletion did not exceed 1.4 Mb in size and was either atypical or type-1 but had breakpoints lying outside of the known hotspot regions PRS1 and PRS2 (Supp. Fig. S1). Patient 1860-M has an atypical 1.3 Mb *NF1* deletion as demonstrated in our previous study [Steinmann et al., 2008] (Supp. Fig. S2). FISH analysis indicated that the number of cells harboring the atypical *NF1* deletion was very high in uncultured whole blood of patient 1860-M (96%, Table 1). By contrast, patient 3304 exhibited much lower levels of del(+/–) cells in uncultured blood (17%). In both patients, the proportion of del(+/–) cells in different leukocyte subtypes was similar to the proportions detected in whole blood samples (Table 1).

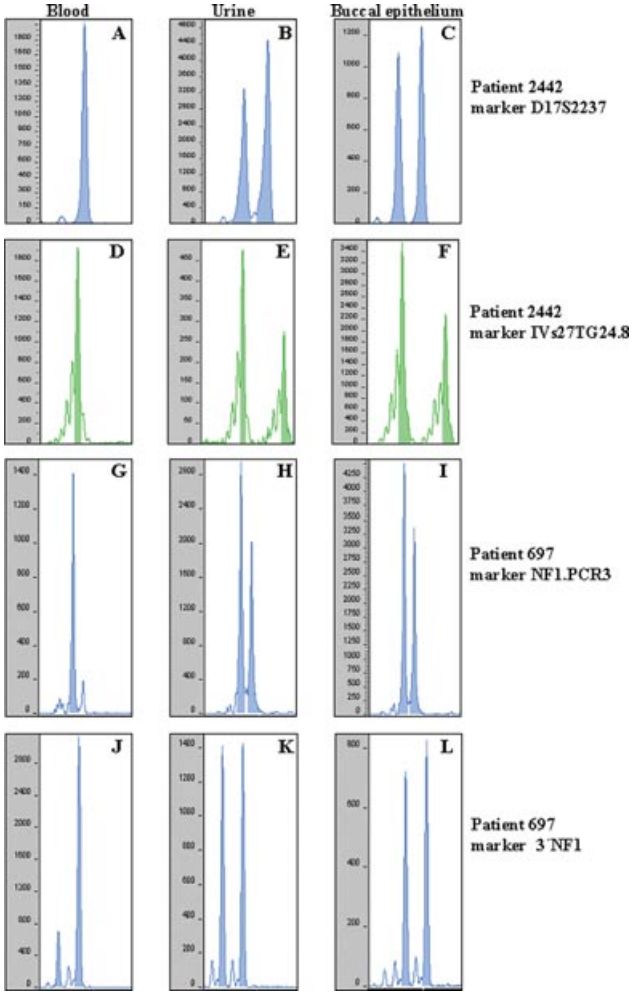

Figure 1. Microsatellite marker analysis using DNA extracted from uncultured whole blood, urine, and buccal epithelium from patients 2442 and 697 who both possess mosaic type-2 *NF1* deletions. The markers analyzed are located within the type-2 *NF1* deletion region. **A–F:** In the blood of patient 2442, only one allele of markers D17S2237 and IVs27TG24.8 was detected owing to the high proportion of del(+/–) cells. However, heterozygosity of these markers was clearly observed in both urine and buccal epithelium, since the proportion of normal cells was much higher in these tissues. **G–L:** In the blood of patient 697, only small peaks of the second alleles were visible. In urine and buccal epithelium, however, heterozygosity was obvious, clearly indicating mosaicism in this patient.

FISH and Microsatellite Marker Analysis of Urine-Derived Epithelial Cells from Patients with Mosaic *NF1* Deletions

Mosaicism with normal cells is difficult to assess with any degree of certainty when the number of del(+/–) cells in blood exceeds 98%. Therefore, we sought an additional tissue or cell type that could be tested in order to improve the analysis of somatic mosaicism in patients with high proportions of blood del(+/–) cells. In our previous analysis [Steinmann et al., 2007], we performed FISH on buccal epithelial cells. However, the slide quality was often poor and hence the FISH results proved difficult to evaluate. In this study, we assessed whether epithelial cells from urine samples of patients with mosaic *NF1* deletions might be more appropriate for the analysis of mosaicism by FISH. Using urine samples, the error rate of the FISH analysis regarding the number of false-positive normal cells was low, on average 0.2% (Supp. Table S7). Intriguingly, the proportions of del(+/–) cells were considerably lower in urine samples (23.9–81.9%) from patients with mosaic type-2 *NF1* deletions as compared with uncultured blood (93.8–99%, Table 1). Similarly, in patient 1,860-M with a mosaic atypical *NF1* deletion detected in 96% of uncultured whole blood cells, a relatively low proportion of del(+/–) cells was observed in urine (51%, Table 1). We therefore conclude that uroepithelial cells are most appropriate as a means to assess mosaicism with normal cells in patients with high numbers of del(+/–) cells in blood. The vast majority of cells from the urine samples that we analyzed by FISH were squamous epithelial cells with polygonal appearance as determined by microscopic inspection.

Microsatellite marker analysis of DNA from urine-derived epithelial cells also proved to be very useful in detecting mosaicism with normal cells (Fig. 1, Supp. Table S12). For example, in patient 2442, the deletion was detected in 99.3% of the uncultured blood cells and consequently, only one allele derived from markers located within the type-2 deletion interval was observed in blood-derived DNA (Fig. 1A and D). However, in urine and buccal epithelium, marker heterozygosity was detected owing to the higher number of normal cells found in these tissues (Fig. 1B–F). Comparative microsatellite marker analysis was also helpful in interpreting small marker peaks detected in blood. For example, a second small marker peak was noted in blood from patient 697, possibly indicative of marker heterozygosity (Fig. 1G and J). Such small peaks are, however, difficult

Table 3. FISH Results of Skin Fibroblasts and Cutaneous Neurofibroma (nf) Derived Cells from Patient 488 Harboring a Mosaic Type-2 *NF1* Deletion

Cells analyzed	Proportion of del(+/–) cells (%)
Fibroblasts from skin overlying an nf on the trunk[a]	39.2
Fibroblasts from skin overlying an nf on the head[a]	50.4
Fibroblasts from skin overlying an nf on the neck[a]	49.7
Cells of an nf from the trunk[b]	77.9
Cells of an nf from the head[b]	79.3
Cells of an nf from the neck[b]	91.4

[a] Fibroblasts were derived from skin overlying different neurofibromas as indicated. The skin fibroblasts were cultured for 51 days and then prepared for FISH analysis.
[b] Cells derived from cutaneous neurofibromas were directly prepared for FISH analysis after dissociation of cells from tumor tissue.

to interpret since "stutter" peaks are a common problem inherent to the analysis of dinucleotide repeat markers. However, the comparison of the height of the marker alleles observed in urine and buccal epithelium with those in the blood of patient 697 clearly identified the second allele, unequivocally indicating marker heterozygosity due to the higher proportion of normal cells in urine and buccal epithelium (Fig. 1H–L).

Taken together, microsatellite marker analysis of urine DNA samples from 11 patients with mosaic type-2 *NF1* deletions detected marker heterozygosity and hence mosaicism with normal cells, in all of them (Supp. Figs. S4–S12; Supp. Table S12). Thus, microsatellite marker analysis using DNA derived from urine samples could in principle replace FISH as a means to assess mosaicism with normal cells in patients with type-2 deletions or any other large *NF1* deletion with high proportions of del(+/–) cells detected in blood.

FISH Analysis of Skin Fibroblasts and Neurofibroma-Derived Cells

In patient 488, 99% of the blood cells exhibited the type-2 deletion (Table 1). Much lower proportions of del(+/–) cells were detected not only in urine but also in fibroblast cultures from skin samples overlying neurofibromas (39–50%; Table 3). In neurofibroma derived cells, however, the proportion of del(+/–) cells was on average 83% and thus higher than in skin fibroblasts that exhibited an average proportion of del(+/–) cells of 46%.

Proportions of Cells with Mosaic Type-2 Deletions in Blood as Compared to Mosaic Atypical or Type-1 *NF1* Deletions

Six patients with generalized (non segmental) NF1 and mosaic atypical or type-1 *NF1* deletions have been previously reported, and the proportion of del(+/–) cells in blood of these patients has been investigated by FISH [Maertens et al., 2007; Messiaen et al., 2011; Streubel et al., 1999; Tinschert et al., 2000; Tonsgard et al., 1997; Wu et al., 1997] (listed in Supp. Table S13). Including patients 3304 and 1860-M analyzed here, the proportions of del(+/–) cells are now known for a total of eight patients with mosaic non-type-2 *NF1* deletions. Remarkably, only two of these eight patients exhibited proportions of del(+/–) cells in blood of >90%. By contrast, all 11 patients with type-2 *NF1* deletions investigated in this study had proportions of del(+/–) cells in blood of >90% (Table 1). Hence, high proportions of del(+/–) cells (>90%) are significantly more frequent in patients with type-2 deletions than in patients with other types of mosaic *NF1* deletions ($P = 0.001$, two-tailed Fisher's exact test). Even if the two patients reported by Tonsgard et al. [1997] and Wu et al. [1997], who have not been well characterized with respect to the extent of the respective mosaic deletion, were to be excluded from the analysis, this difference would remain statistically significant ($P = 0.006$, two-tailed Fisher's exact test).

X-Inactivation in Patients with Mosaic *NF1* Deletions

The XCI pattern in nine female patients with mosaic *NF1* deletions was investigated by means of the *HUMARA* methylation assay. Eight of these nine patients had type-2 deletions whereas one patient (1860-M) had an atypical *NF1* deletion. In seven of these nine females, skewed XCI (≥75% of the predominant allele) was observed in blood. In six of these seven patients with mosaic *NF1* deletions and skewed XCI in blood, we observed random XCI in buccal epithelium or urine (Table 4; Supp. Table S14). In patient 938, skewed XCI was noted not only in blood but also in buccal epithelium and urine, suggesting that primary skewing may affect all tissues in this patient. In the blood of patient 1502, we observed an XCI ratio of 28:72, which just fell short of meeting the diagnostic criterion for skewed XCI. By convention, skewing is considered only if ≥75% of the cells inactivate the same X chromosome. In patient 488, who possesses a mosaic type-2 deletion, the XCI pattern was clearly random in blood (54:46). Random XCI was also observed

Table 4. X Chromosome Inactivation Pattern (XCIP) Determined by the *HUMARA* Methylation Test in 8 Patients with Mosaic Type-2 *NF1* Deletions, in Patient 1860-M with a Mosaic Atypical *NF1* Deletion, and Patient 2358 with a Non-Mosaic Type-2 Deletion

	Proportion (%) of cells with the deletion in		*AR* allele ratio A1:A2 (%) in			
Patient (age in years) [deletion]	Blood[a]	Urine	Blood	Buccal epithelium	Urine	XCIP
697 (19) [mosaic type-2]	98.7	27.6	15:85	34:66	nd	Skewed in blood; random in buccal epithelium
736 (76) [mosaic type-2]	96.8	26.7	15:85	56:44	nd	Skewed in blood; random in buccal epithelium
UC172 (10) [mosaic type-2]	93.8	64.5	5:95	51:49	nd	Skewed in blood; random in buccal epithelium
1630 (19) [mosaic type-2]	96.5	29.3	10:90	29:71	nd	Skewed in blood; random in buccal epithelium
2442 (42) [mosaic type-2]	99.3	51.7	21:79	28:72	nd	Skewed in blood; random in buccal epithelium
1860-M (54) [mosaic atypical]	95.7	50.9	82:18	n.d.	65:35	Skewed in blood; random in urine
938 (39) [mosaic type-2]	97.9	23.9	19:81	10:90	19:81	Constitutive skewing
488 (41) [mosaic type-2]	99.0	46.5	54:46	19:81	63:37	Random in blood and urine; skewed in buccal epithelium
1502 (30) [mosaic type-2]	95.9	54.6	28:72	71:29	nd	Random in blood and buccal epithelium
2358 (13) [non-mosaic type-2]	99.4	99	41:59	38:62	nd	Random in blood and buccal epithelium

[a] Blood cells were directly prepared for FISH analysis without prior culturing of blood cells.
nd, not determined.
The ratio of the CAG-repeat alleles within exon 1 of the androgen-receptor (*AR*) gene is indicated as well as the proportions of del(+/–) cells as determined by FISH. Skewed XCI is considered if the proportion of the predominant *AR* allele on the inactive X chromosome ≥ 75%.

in the blood of patient 2358 who possessed a non-mosaic type-2 deletion.

Discussion

In this study, we analyzed mosaicism in 11 patients with type-2 *NF1* deletions and two patients with atypical or type-1 deletions. High proportions (94–99%) of cells harboring the deletion [del(+/–) cells] were evident in the blood of all 11 patients with type-2 deletions (Table 1). Nine of these 11 patients had already been investigated several years ago at which time high rates of del(+/–) cells had been evident in blood (Supp. Table S10). Hence, the proportion of del(+/–) cells appears to be consistently high over time in the blood of patients with type-2 *NF1* deletions. In contrast to blood, much lower proportions of del(+/–) cells (24–82%) were evident in urine from the 11 patients with type-2 deletions. However, these tissue-specific differences in the proportion of del(+/–) cells were not found to be exclusive to patients with type-2 deletions. Patient 1860-M, who possessed an atypical large *NF1* deletion, also exhibited a much higher proportion of cells with the deletion in blood (96%) than in urine (51%; Table 1). We conclude that type-2 *NF1* deletions (and rarely also other types of *NF1* deletion) arise at an early stage during embryonic development and that hematopoietic primordial stem cells harboring these deletions may have a selective growth advantage over normal stem cells lacking the deletion. The type-2 deletions investigated in this study, as well as the atypical deletion of patient 1860-M, must have occurred during the blastocyst stage or even earlier, certainly before embryonic day 12 and the onset of gastrulation because del(+/–) cells were detected in derivatives of all three germ cell layers, the endoderm (epithelial cells from the urinary system), mesoderm (blood cells), and ectoderm (skin fibroblasts, neurofibroma-derived cells, and buccal epithelium) (Supp. Table S15).

Further conclusions about the timing of the deletion event in female patients with mosaic *NF1* deletions may be drawn from the XCI pattern. XCI is initiated in preimplantation blastocysts [Okamoto et al., 2011; van den Berg et al., 2009, 2011]. Subsequently, chromosome-wide XCI gradually becomes fully established during implantation and the postimplantation blastocyst stage [Migeon, 2007; Okamoto et al., 2011]. It has been estimated that 8–16 progenitors of pluripotent hematopoietic stem cells are present at the time of XCI [Amos-Landgraf et al., 2006; Fialkow, 1973; Prchal et al., 1996; Puck et al., 1992]. During primary XCI, the choice of the X chromosome to be inactivated occurs mostly at random. However, significant deviations from the theoretical 50:50 XCI ratio of the two parental X chromosomal alleles have been detected in healthy females. Such a "skewed" XCI pattern is considered if ≥75% of the cells inactivate the same parental X chromosome [Bolduc et al., 2008; Busque et al., 2006]. The incidence of skewing in healthy females is known to vary with age and between different tissues [Amos-Landgraf et al., 2006; Bolduc et al., 2008; Busque et al., 2006; Sandovici et al., 2004; Sharp et al., 2000]. Approximately 28% of healthy females <43 years exhibit skewed XCI in blood [Bolduc et al., 2008]. However, the incidence of skewing increases to 40% in the blood of healthy women >60 years [Busque et al., 2006]. When we consider only those seven of the nine investigated females with mosaic *NF1* deletions who were younger than 43 years, three of these seven patients were found to exhibit moderately skewed XCI (≥75% and ≤91% of the predominant allele) in blood but not in epithelial tissues, whereas two patients exhibited random XCI in blood (Table 4). In one patient (UC172) extremely skewed XCI was observed in blood (≥91% of the predominant allele) but not in buccal epithelial cells. We conclude that in this patient, the deletion may have occurred after XCI as schematically depicted in Figure 2A. By contrast, the XCI patterns observed in the other patients with mosaic *NF1* deletions imply that the respective deletions occurred before the onset of XCI.

In contrast to patients with type-2 *NF1* deletions and patient 1860-M with an atypical deletion, patient 3304 (who possesses a mosaic type-l or atypical *NF1* deletion) was found to exhibit a low rate of the deletion in both blood (17%) and urine (25%; Table 1). Although the rates of the deletion were low, the fact that cells of both mesodermal and endodermal origin harbored the deletion indicate an early occurrence of the deletion during embryonic development in this patient as well. Since patient 3304 had numerous cutaneous neurofibromas (Supp. Table S3), ectodermal cells are also affected by the deletion. We conclude that also the deletion in patient 3304 should have preceded gastrulation. However, his deletion probably occurred later than the deletions in the other mosaic patients investigated here because of the low proportions of the del(+/–) cells. The lack of a growth advantage of hematopoietic del(+/–) stem cells in this patient may be related to the relatively late occurrence of the deletion.

Taken together, our findings imply that mosaic *NF1* deletions, which are present in a high proportion of peripheral blood cells of affected NF1 patients, arise early during embryonic development. The majority of them is likely to have occurred in the preimplantation blastocyst stage or even earlier, before the onset of XCI. A selective advantage of hematopoietic del(+/–) stem cells present during a specific window of embryonic development could then account for the observed tissue-specific differences in the proportions of cells harboring the deletions. *NF1* is known to act as a tumor suppressor gene in hematopoietic progenitor cells and loss of *NF1* heterozygosity is a cause of juvenile myelomonocytic leukaemia (JMML) [Side et al., 1997; Steinemann et al., 2010]. One of the hallmark features of JMML is the hypersensitivity of myeloid progenitor cells to granulocyte-macrophage colony stimulating factor (GM-CSF) in colony forming assays [Emanuel et al., 1991]. The *NF1* gene product, neurofibromin, acts as a negative regulator of Ras and hyperactive Ras/mitogen-activated protein kinase (MAPK) signalling is known to be causally involved in the pathogenesis of JMML and other myeloid cancers [reviewed by Loh, 2011]. Children (but not adults) with NF1 are at a >200-fold increased risk of JMML [Bader and Miller, 1978]. Intriguingly, the risk of JMML in patients with NF1 appears to be confined to a narrow developmental window. Those children with NF1 who do not develop JMML during their first few years are not at any increased risk of developing hematologic malignancies later in life. Thus, neurofibromin is an essential Ras GTPase-activating protein in hematopoietic stem cells that are hypersensitive towards *NF1* loss during a specific time window of development.

Adoptive transfer of $Nf1^{-/-}$ liver cells into irradiated wild-type recipient mice results in a myeloproliferative disease [Bollag et al., 1996; Largaespada et al., 1996]. Moreover, *Nf1*-deficient myeloid and lymphoid stem cells are known to exhibit a growth advantage in competitive repopulation studies of irradiated recipient mice [Zhang et al., 2001]. Taken together, these observations have suggested a key role for neurofibromin not only in myeloid cells but also in lymphocyte development and function. Biallelic *NF1* inactivation has been observed in a small subset of children with acute myelogenous leukemia (AML) and T-cell acute lymphoblastic leukemia (T-ALL) [Balgobind et al., 2008]. Furthermore, somatic *NF1* deletions with a minimal deleted region of 0.9 Mb have been identified in 11% of adults with AML [Parkin et al., 2010]. These findings imply that loss of neurofibromin is an important

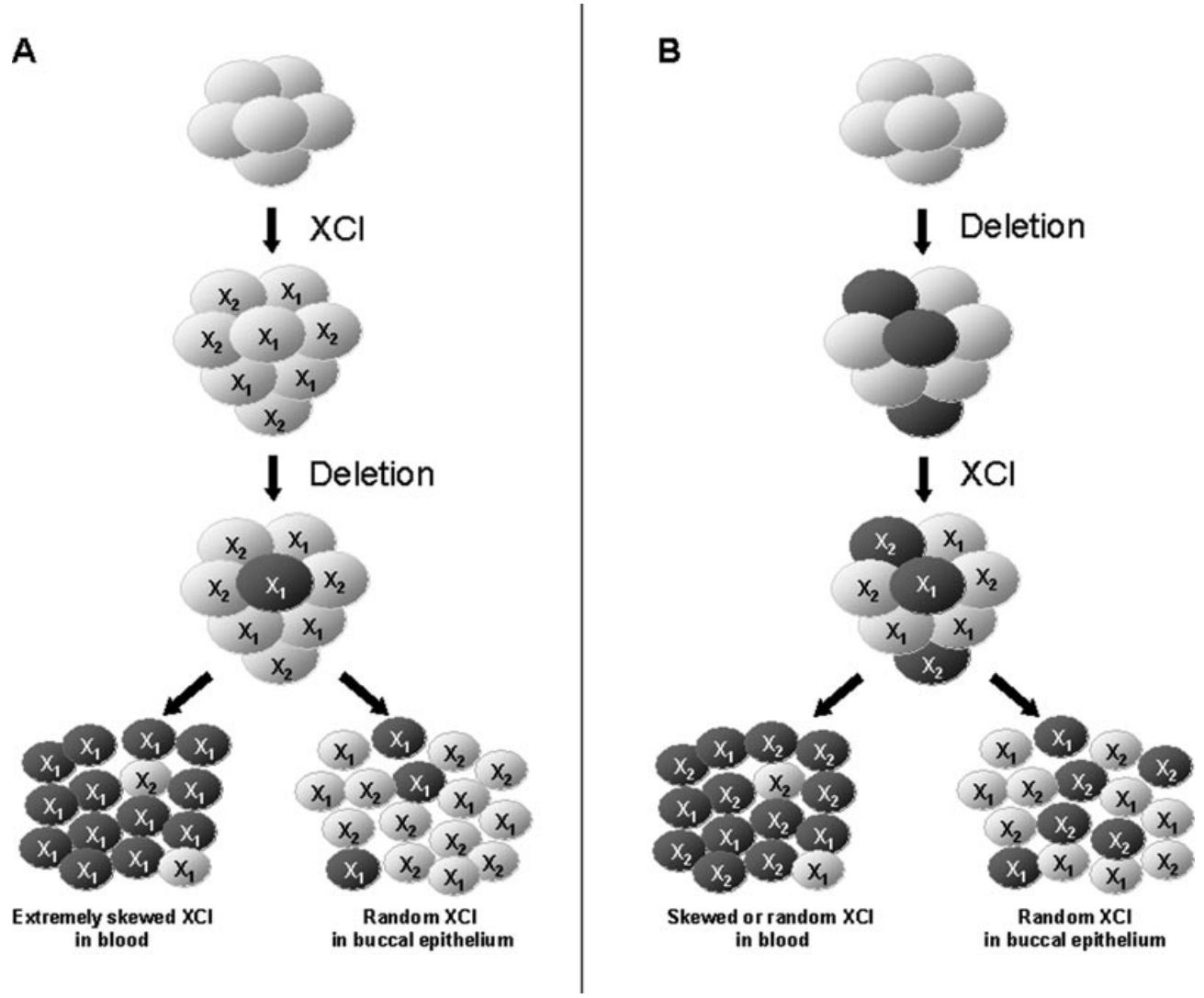

Figure 2. Model to explain the consequences of X chromosome inactivation (XCI) preceding the deletion event (or vice versa) as well as the consequences of a selective growth advantage of hematopoietic stem cells harboring the deletion (del(+/–) cells) over normal cells in patients with mosaic *NF1* deletions. **A:** If XCI precedes the deletion event (indicated by a dark gray color of the cells), all del(+/–) cells will have the same X chromosomal *AR* allele inactivated. The onset of XCI, which occurs randomly, is indicated by X_1 or X_2. Owing to the selective advantage of primordial hematopoietic del(+/–) stem cells over normal cells, extremely skewed XCI ($\geq$91% of the predominant allele) is observed in blood. In buccal epithelium, del(+/–) cells exhibit no growth advantage over normal cells and hence, much lower proportions of del(+/–) cells are detected as well as random XCI. This model may explain the extremely skewed X inactivation observed in blood of patient UC172. **B:** If the deletion precedes XCI, random or mildly skewed XCI ($\geq$75% but $\leq$91% of the predominant allele) is observed in blood of patients with large *NF1* deletions. Owing to the selective advantage of primordial hematopoietic del(+/–) stem cells over normal cells, high rates of del(+/–) cells are observed in blood of the patients. According to this model, the deletions occurred during the preimplantation blastocyst stage or even earlier when both X chromosomes (X_1 and X_2) were active. Owing to the selective advantage of primordial hematopoietic del(+/–) stem cells over normal cells, high rates of del(+/–) cells are observed in blood of the patients resulting in random or skewed XCI. By contrast, epithelial del(+/–) stem cells do not exhibit selective growth over normal cells. Hence, random XCI is noted in urine or buccal epithelial cells. This model may explain the XCI pattern as well as the rates of del(+/–) cells observed in patients 697, 1630, 736, 2442, 1860, 488, and 1502.

mechanism in hematologic malignancies other than JMML [Balgobind et al., 2008; Parkin et al. 2010]. Nevertheless, an increased risk to NF1 patients of hematologic malignancies other than JMML, either with intragenic mutations or large *NF1* deletions, has not so far been reported.

In mice, the homozygous inactivation of *Nf1*, and even the loss of a single *Nf1* allele, is sufficient to modulate Ras activity in unstimulated T-cells, and this correlates with a substantial increase in T-cell subset numbers [Ingram et al., 2002]. Alongside the various other lines of evidence outlined above, these data indicate that neurofibromin is an important growth regulator of hematopoietic stem cells in both the myeloid and lymphoid compartments. We surmise that *NF1* haploinsufficiency consequent to a large *NF1* deletion in susceptible primordial hematopoietic stem cells confers a growth advantage upon del(+/–) stem cells over normal stem cells lacking the deletion. Our findings in NF1 patients harboring mosaic *NF1* deletions suggest that this growth advantage results in high proportions of del(+/–) cells in peripheral blood. Importantly, we observed high levels of the deletions in all leukocyte subtypes investigated (Table 1). Since the proportions of cells containing the deletion were

high in all leukocyte subtypes analyzed, the separation of leukocyte subtypes and subsequent FISH analysis failed to improve the detection of low rates of normal cells as frequently observed in patients with mosaic type-2 deletions. Our present study has however indicated that FISH analysis of urine-derived epithelial cells or microsatellite marker analysis in urine-derived or buccal epithelial cells represents excellent methods to detect mosaicism in patients with large *NF1* deletions.

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