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ORIGINAL ARTICLE

Haplotype-assisted characterization of germline mutations at short tandem repeat loci

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Abstract In this study, 98 families with 101 mutations were analyzed in depth in which a mutation had been observed at one of the four loci D3S1358, FGA, ACTBP2, and VWA. To determine the origin (male/female) of the mutation, five to seven polymorphic flanking markers were selected for each locus concerned and used to construct family-specific haplotypes. Additionally, all alleles of the STR system concerned were sequenced. With this duplicate approach, it was possible to identify the mutated structure and/or mutation event in the vast majority of cases. The ratio of one-step to two-step mutations was 100:1. The ratio of paternal to maternal mutations was 76:8. The ratio of gains to losses was 47:50. Also, the mutation rates in two systems, ACTBP2 and VWA, were clearly higher than those given in the literature.

Keywords Mutation · Short tandem repeat · Flanking marker · Haplotype

Introduction

Germline mutations at short tandem repeat loci (STRs) are observed as gains or losses of repeat units. The mainly accepted mechanism for these mutations is slipped strand mispairing [1–5], although the model of unequal crossing over (UEC) exists furthermore [6, 7].

Microsatellites are often applied to investigate paternity cases [8–15]. Typically, a set consisting of approximately 15 STRs is applied [16–22]. If an isolated mismatch occurs in such case work, a mutation event is assumed and the alternative would be exclusion. Therefore, the possibility of the mutation must be carefully analyzed before a conclusion can be reached [2, 8, 9, 23–27].

Referring to a previous paper [2], the parameters (1) origin (male/female), (2) size (one-step/two-step or other), and (3) sequence structure are usually analyzed to categorize a mutation, and the principles (1) paternal>maternal, (2) single-step>two-step, and (3) integer mutational steps are widely accepted.

However, there exist cases in which the classification is not that easy and clear. For instance, the new allele can have originated either from the father or the mother (e.g., child 15/17 and both parents 14/17). Also, the possibility of, e.g., a maternal one-step mutation can concur with a paternal two-step mutation (or even more). In addition, there also exist examples of hitherto unknown mutation types concurring with exclusion (unpublished data).

Therefore, we have carried out analyses to classify the mutation. Klintschar et al. [5] constructed haplotypes by using flanking markers to the STR locus in question. With the same objective — classification of the mutation event — we chose from our mutation material 98 cases with 101 mutations where the event was assigned to one of the four STR loci D3S1358, FGA, ACTBP2 (= SE33), or VWA. These four loci, which are all included in the German DNA database, were selected because mutations are more common [2, 28–31]; the cases were randomly chosen. In three of these 98 cases, two children of the respective families showed a mutation in the same STR system; the

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STR system FGA was involved once and the ACTBP2 system twice (pedigrees F-10, A-7, and A-18/Fig. S1).

Materials and methods

The following loci were selected:

- D3S1358 (chromosomal localization: 3p21.31) shows a simple repeat structure:
  5′-FR-TCTA-$(TCTG)_2$-$(TCTA)_{9–11}$-FR-3′ (alleles 12–14)
  5′-FR-TCTA-$(TCTG)_3$-$(TCTA)_{11–15}$-FR-3′ (alleles 15–19)
  5′-FR-TCTA-$(TCTG)_2$-$(TCTA)_{12–14}$-FR-3′ (alleles 15′–17′),
  FR=flanking region. The heterozygosity index is 0.78 [32], and the mutation rate is in the range of 0.12% [33] (STRbase; http://www.cstl.nist.gov/biotech/strbase/). We report on 22 cases/22 mutations.
- FGA (= FIBRA; chromosomal localization: 4q31.3) has about 50 different alleles. The common alleles show a compound structure:
  5′-FR-$(TTTC)_3$-TTTT-TTCT-$(CTTT)_{10–19}$-CTCC-$(TTCC)_2$-FR-3' (alleles 18–27)
  5′-FR-$(TTTC)_3$-TTTT-TT-$(CTTT)_{15–16}$-CTCC-$(TTCC)_2$-FR-3' (alleles 22.2, 23.2).
  The heterozygosity index is 0.86 [34], and the mutation rate is approximately 0.28% [33] (STRbase). We analyzed 40 cases/41 mutations.
- ACTBP2 (=SE33; chromosomal localization: 6q14 [35]) is one of the most informative markers used in forensic genetics. The sequence structure is compound:
  5′-FR-$(AAAG)_{12–22}$-FR-3′ (alleles 12–22)
  5′-FR-$(AAAG)_{5–16}$-AAAAAG-$(AAAG)_{8–23}$-FR-3′ (alleles 19.2–35.2); also alleles with two interposed hexamers occur [36–38].The heterozygosity index is 0.94 [39], and the mutation rate is in the range of 0.64% [33] (STRbase). We report on 24 cases/26 mutations.
- VWA (chromosomal localization: 12p13.31) shows the following repeat structure:
  5′-FR-TCTA-$(TCTG)_4$-$(TCTA)_{8–17}$-FR-3′ (alleles 13, 15–22)
  5′-FR-TCTA-TCTG-TCTA-$(TCTG)_4$-$(TCTA)_3$-TCCA-$(TCTG)_3$-FR-3′ (rare allele 14).
  The heterozygosity index is 0.73 [40], and the mutation rate is approximately 0.17% [33] (STRbase). We report on 12 cases/12 mutations.

DNA from buccal smears was extracted using a modified Chelex–proteinase K method [41], and the profiles were typed using different multiplex kits (e.g., Identifiler, SEfiler, Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The parenthood was, without exception, regarded as proven with a probability value $W \geq 99.99\%$, even including the mutation. If this threshold was not reached up to 16, additional STR systems were added in a second approach. Altogether, 98 cases comprising 101 mutations were investigated.

Selection criteria of these flanking markers were the genetic distance (approximately 8 cM) and high polymorphism. Via diverse internet sources (e.g., http://www.gdb.org, http://genecards.weizmann.ac.il, etc.) and publications, five to seven polymorphic flanking markers upstream as well as downstream were selected for each of the four loci, the relationship between both sides being roughly 50:50 (Table S1) (e.g., [35, 42]).

The flanking markers, their distance to the respective STR system holding the mutation, the primer sequences, and the repeat motifs are shown in detail in Table S1.

Each amplicon was regarded as an "allele" and thus used to construct family-specific haplotypes as described by Klintschar et al. [5] (Fig. 1 and Fig. S2). The notation of the amplicons was arbitrary as follows (see Table S2): all amplicons observed in a given system were arranged according to their sizes and then arbitrarily and consecutively numbered. The nomenclature is, therefore, not repeat-based.

Exceptions are the amplicons of the systems "STR1" and "STR2" which are located next to the VWA locus [40] and the forensically established STR system D12S391 [43] where the allele numbering is in accordance with the repeat number.

Using these flanking markers, family-specific haplotypes could be constructed for many cases (Table S3), and thus, the type of mutation elucidated.

Results and discussion

We have analyzed 98 families with 101 mutations in their offspring. Among them there existed three with more than one mutation in the systems investigated. One additional mutation has not been included because it occurred in an identical twin. Of the families, 61 had more than one child and could therefore be investigated using the "haplotype approach" ("HA") which was successful in 53 of them. Furthermore, the "allelic structure approach" (ASA) was also applied, and the mutated allelic structure in 72 of the total of families investigated could be identified (Table 1).

If there was only one child, the HA failed by definition (Fig. 2). This occurred in 40 families. These were nevertheless "haplotyped" to enlarge the dataset of flanking markers for eventual statistical evaluation. The ASA enabled the assignment of the mutation to its origin and thus to the mechanism in 27 of them. The informative value of the sequenced alleles is very high, especially for alleles with a compound structure, e.g., ACTBP2 (=SE33). Almost 68% of the one-child cases could therefore be solved by sequencing.

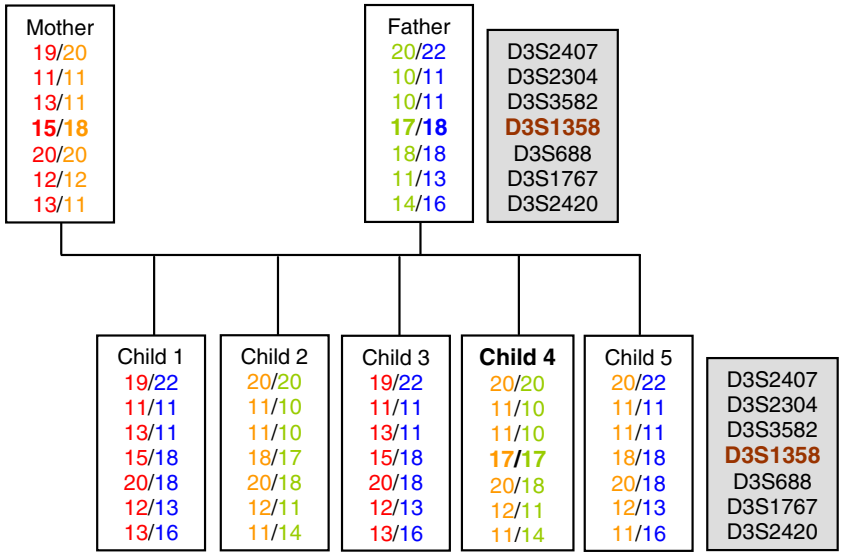

Fig. 1 The maternal haplotypes are shown in *red* and *orange*, the paternal in *green* and *blue*. The child holding the mutation is written in *bold letters*. The child's mutation could be identified as maternal, one-step deletion (18→17)

The following alternatives were not considered in our allelic assignment approach:

1. Mutations in the alleles from both parents (in contrast to only one)
2. A three-(or more)-step mutation (in relation to a one-step mutation). This (theoretical) alternative was a possibility in 18 cases
3. A mutation resulting in affected alleles with incomplete repeat elements (hereinafter referred to as "uneven mutation", e.g., 7.2 repeat units) instead of the one-step standard (four such cases, see for instance pedigree A-17/Fig. S1)

Altogether, the mutated allelic structure was identified in 97 mutations; among them, there remained 17 cases with an unclear origin (paternal/maternal) because both alleles could have mutated with an equal chance and with the same allelic structure. There remained three mutations where the direction, i.e., gain or loss, was unclear, and thus, also the mutated allelic structure, but the origin and the magnitude (one-step) could be ascertained. There was one case where neither the origin (maternal vs. paternal) nor the direction could be stated but only the magnitude (one-step vs. two-step; 1st VWA case/Table S4).

There were also cases with several children showing an inappropriate case constellation which could not be solved with the help of the flanking markers selected (Fig. 3).

There occurred only one two-step mutation (see pedigree A-15/Fig. S1) in relation to 100 one-step mutations. The gender ratio, i.e., paternal/maternal was 76:8. This nearly ten-fold higher paternal to maternal mutation rate was already observed and described in previous studies [2, 28, 44]. Gains and losses were fairly balanced (47:50).

The results are consistent with the slipped strand mispairing theory caused by polymerase slippage. This theory delivers the best explanation for the observed de novo mutations [2]. The fact that 100 out of 101 analyzed mutations were categorized as one-step mutations is more in line with this model [45, 46].

Table 1 Summary of the obtained results

	Classification of the analyzed mutation via combination of ASA and HA	Mutation identified via ASA/verified via HA	Mutation identified via HA	Origin undetermined	Effect undetermined	Origin+effect undetermined
ACTBP2 (= SE33)	25 of 26	23/19	2	1[a]		
D3S1358	15 of 22	15/8		6	1	
VWA	9 of 12	5/4	4	1	1	1
FGA	32 of 41	29/14	3	8	1	
Total	81 of 101	72/44	9	16	3	1

HA Haplotype approach, *ASA* allelic structure approach

[a] Two-step mutation

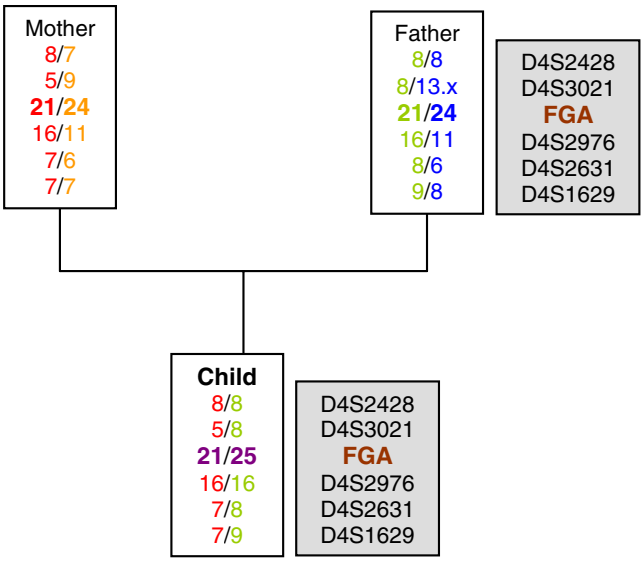

Fig. 2 Mutations from single-child families are inappropriate to be solved by flanking markers because the parental haplotypes cannot be determined for sure

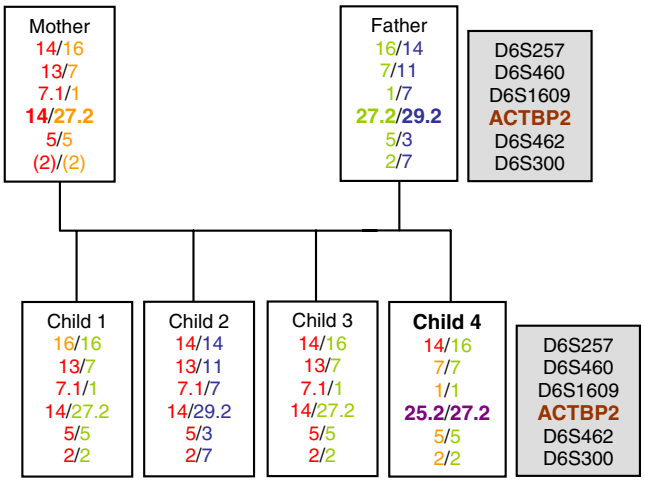

Fig. 4 Due to the special allele constellation, the filial mutation (27.2→25.2) could not be assigned to a particular parent, although the paternal haplotypes could be unequivocally reconstructed. At least, the mutation was shown to be a two-step mutation

A rare two-step mutation was observed in only one case (see Fig. 4). Although this de novo mutation could not be assigned to either parent, it was clearly a two-step deletion.

Also, the original estimate of a gender ratio of 1:6 (maternal/paternal) [2] possibly requires correction, i.e., to nearly 1:10. These differences are obviously due to the much lower figures in previous publications [2]. Also, the present ratio will possibly require further adjustment.

The ratio of gains and losses is balanced; this could be regarded as a hint: STR mutations by replication slippage do not appear to be in favor of one or the other direction. Therefore, the assumption of a "shriveling genome" [2] could not be confirmed by our current data.

Summing up, the flanking markers can be indicated as a useful tool to characterize de novo mutations — as an alternative or in addition to conventional sequencing. By definition, the HA can only be successful in families with more than one child. Nearly 90% of the examined mutations could be unequivocally categorized, i.e., with respect to origin, effect, degree, and mechanism.

Hence, it was shown that a one-step mutation has the highest priority in categorizing mutations (100:1). Subsequently, a paternal origin is more likely than a maternal

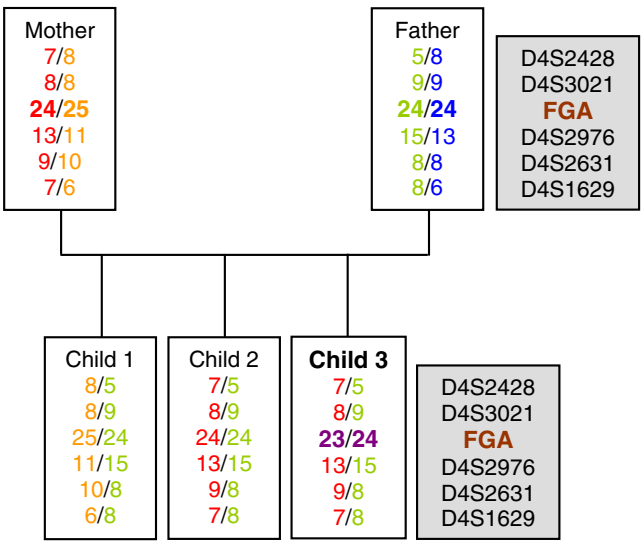

Fig. 3 Although, due to the siblings, the definite parental haplotypes could be determined, it is impossible to classify the mutation with the help of flanking markers

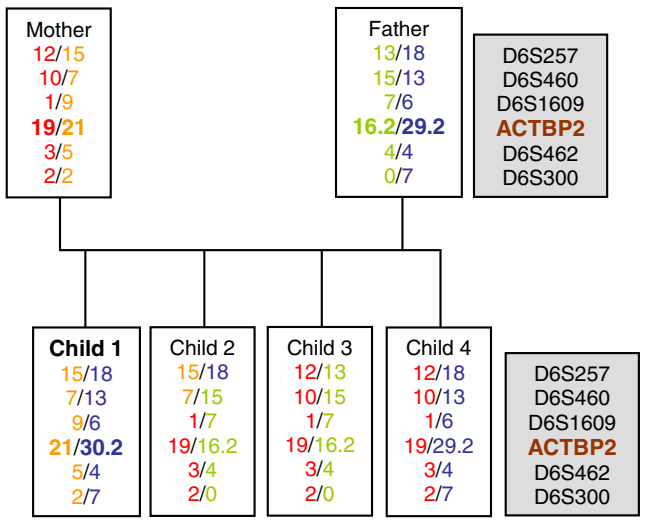

Fig. 5 Child 2 shows upstream from D6S460 alleles from the other maternal haplotype, and upstream from D6S257, the different paternal haplotype caused by crossing over

(10:1). The mutation direction, i.e., gains or losses, was fairly balanced.

Taken from the data, it can be stated that two-step mutations seem to be much rarer than originally assumed, and their proportion is roughly 1%.

No hints were observed suggesting UEC. This fact was already observed earlier [5] and confirmed by our study. Using flanking markers, crossing overs were occasionally detected, but a UEC was not among them.

Hypothetically, some cases in our series also bore the chance of a multi-step mutation, i.e., three and more steps. But this was not confirmed in a single case. In our opinion, >two-step mutations are extremely rare — if they exist at all. Therefore, we assume that there is no linear correlation between the mutation rates and the ongoing number of steps. If we assume that a two-step mutation is 100 times less frequent than a one-step mutation and a three-step mutation is 100 times less frequent than a two-step mutation, then a three-step mutation would have a frequency that is $100 \times 100 = 10,000$ times smaller than for a one-step mutation. This again would need to be multiplied by the observed mutation rate of, for instance, 0.2% prior to the calculation of the probability index (PI) values. This ratio of 1:5 million needs to be included in the formula used for the calculation of the paternity probability (W value) or the PI value. To counterbalance this a priori and reach the threshold of 99.9%, a high number of STR systems would need to be investigated. At least 20 additional STR systems (on several chromosomes) would need to be analyzed additionally without further exclusion to reach a statistical level of proof. In addition, a consistent sequence structure must be demonstrated regarding the affected alleles. Unless this is performed, the assumption of a multi-step mutation would be very doubtful. In the cases published so far, this approach is missing (e.g., [28]).

The same is true for the alternative assumption of a biparental mutation. If this was assumed in FGA (mutation rate 0.28%, 0.32% paternal and 0.05% maternal), NIST Standard Reference Database (STRbase) http://www.cstl.nist.gov/biotech/strbase), then this combination would be expected to be in a range of roughly 1:500,000. If we compare this to the monoparental alternative, this would be 2,300 times more likely.

"Uneven mutations" have not yet been described. During this study, we observed such a mutation type (unpublished data). Assuming that thousands of mutations have already been observed in laboratories worldwide, this "uneven mutation event" can therefore be taken to occur less frequently than even mutations.

In some cases, one (or more than one) child was showing a new haplotype which could only be explained by one or even more cross over events (Fig. 5). Cross overs occurred more frequently between the STR systems D6S257 and D6S460 which is obviously in relation to the long distance (15.4 cM approximately) of D6S257 to the other flanking markers. Taken together, 51 single and five duplicate cross overs were observed in 49 children out of 101 families.

From our data, we have also calculated the mutation rates by comparing the events with the number of allelic transfers. The mutation rates varied between 0.14% (D3S1358), 0.27% (FGA), 0.89% (SE33), and 0.265% (VWA). While the mutation rates of the loci D3S1358 and FGA are in line with the literature (e.g., NIST Standard Reference Database (STRbase) http://www.cstl.nist.gov/biotech/strbase/), the rates found for ACTBP2 and VWA are definitely higher, i.e., in the range of 50%. The differences are statistically significant and for the time being, we have no idea of the origin.

The fact that FGA and ACTBP2 exhibit the highest mutation rates within the widely used STR loci is not surprising: the loci with the highest mutation rate are the most polymorphic and possess the highest number of alleles [33]. More repeat units give more opportunities for replication slippage [46–48].

All in all, the method was shown to be able to categorize noticeably more mutations without a doubt than using conventional methods [2] even if some rare case constellations were observed, in which the use of flanking markers did not help. Additionally, the flanking markers described are not only a useful tool to classify mutations but also an extra method to confirm parenthood.

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