RESEARCH ARTICLE

Position of Nonmuscle Myosin Heavy Chain IIA (NMMHC-IIA) Mutations Predicts the Natural History of *MYH9*-Related Disease

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MYH9-related disease (MYH9-RD) is a rare autosomal-dominant disorder caused by mutations in *MYH9*, the gene for the heavy chain of nonmuscle myosin IIA (NMMHC-IIA). All patients present from birth with macrothrombocytopenia, but in infancy or adult life, some of them develop sensorineural deafness, presenile cataracts, and/or progressive nephritis leading to end-stage renal failure. No consistent correlations have been identified between the 27 different *MYH9* mutations identified so far and the variable clinical evolution of the disease. We have evaluated 108 consecutive *MYH9*-RD patients belonging to 50 unrelated pedigrees. The risk of noncongenital manifestations associated with different genotypes was estimated over time by event-free survival analysis. We demonstrated that all subjects with mutations in the motor domain of NMMHC-IIA present with severe thrombocytopenia and develop nephritis and deafness before the age of 40 years, while those with mutations in the tail domain have a much lower risk of noncongenital complications and significantly higher platelet counts. We also evaluated the clinical course of patients with mutations in the four most frequently affected residues of NMMHC-IIA (responsible for 70% of *MYH9*-RD cases). We concluded that mutations at residue 1933 do not induce kidney damage or cataracts and cause deafness only in the elderly, those in position 702 result in severe thrombocytopenia and produce nephritis and deafness at a juvenile age, while alterations at residue 1424 or 1841 result in intermediate clinical pictures. These findings are relevant not only to patients' clinical management but also to the elucidation of the pathogenesis of the disease. Hum Mutat 0, 1–9, 2007. **© 2007 Wiley-Liss, Inc.**

KEY WORDS: *MYH9*; nonmuscle myosin IIA; May-Hegglin anomaly; Sebastian syndrome; Fechtner syndrome; Epstein syndrome

INTRODUCTION

May-Hegglin anomaly (MHA; MIM# 155100), Sebastian syndrome (SBS; MIM# 605249), Fechtner syndrome (FTNS; MIM 153640), and Epstein syndrome (EPTS; MIM# 153650) have been considered for a long time to be distinct disorders sharing the common features of autosomal-dominant transmission and thrombocytopenia with giant platelets [May, 1909; Hegglin,

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1945; Epstein et al., 1972; Peterson et al., 1985; Greinacher et al., 1990; Rocca et al., 1993; Mhawech and Saleem, 2000]. The diagnosis of MHA or SBS was made in patients with the only additional finding of granulocyte inclusions similar to the Döhle bodies of infectious diseases, with the distinction between these two conditions lying on the ultrastructural morphology of inclusions [Greinacher et al., 1990; Mhawech and Saleem, 2000]. The diagnosis of FTNS or EPTS was made in subjects presenting with the additional features of kidney impairment and sensorineural deafness with or without leukocyte inclusions and presenile cataracts, respectively [Epstein et al., 1972; Peterson et al., 1985; Rocca et al., 1993; Mhawech and Saleem, 2000]. Macrothrombocytopenia and Döhle-like bodies are present from birth, while kidney, hearing, and ocular defects develop during infancy or adult life. Kidney involvement consists of a progressive nephritis presenting with proteinuria with or without microhematuria. Nephritis often evolves into loss of kidney function and the development of a condition of chronic renal failure (CRF) [Epstein et al., 1972; Peterson et al., 1985; Rocca et al., 1993].

Seven years ago, it was shown that all of these disorders derive from mutations in the *MYH9* gene (MIM♯ 160775), encoding for the heavy chain of nonmuscle myosin IIA (NMMHC-IIA) [Kelley et al., 2000; Seri et al., 2000; Kunishima et al., 2001a; Heath et al., 2001]. Myosin IIA is a conventional, nonsarcomeric myosin expressed in most cells and tissues, where it is involved in several functions including cytokinesis, cell motility, and maintenance of cell shape [Robinson and Spudich, 2000; Sellers, 2000; Wei and Adelstein, 2000; Jacobelli et al., 2004]. The NMMHC-IIA is structured in two domains. The 836 N-terminal residues constitute the motor domain (MD), which forms the myosin globular head responsible for ATPase and actin-binding activity and the neck participating in forces amplification, light chain binding, and regulation. The remainder of the molecule constitutes the tail domain (TD), which is responsible for both dimerization of heavy chains in coiled-coil structures and association of myosin molecules into functional filaments [Sellers, 2000]. To date, 27 different *MYH9* mutations have been identified [Kelley et al., 2000; Lalwani et al., 2000; Seri et al., 2000, 2003; Heath et al., 2001; Kunishima et al., 2001a, 2001b, 2003; Arrondel et al., 2002; Ma et al., 2006; Otsubo et al., 2006; Schleinitz et al., 2006]. In most cases they are missense mutations affecting either the MD or the TD of NMMHC-IIA. In some families, the disease derives from nonsense or frameshift alterations located in the last exon, encoding for the C-terminus of the TD. Therefore, it has been hypothesized that MHA, SBS, FTNS, and EPTS are allelic disorders, but the study of three different case series failed to identify consistent genotype–phenotype correlations and showed that the same *MYH9* mutations could be associated with different clinical pictures, even in patients belonging to the same pedigree. Thus, it has been concluded that MHA, SBS, FTNS, and EPTS are not distinct entities but rather they represent different clinical expressions of a single disease, whose phenotype results from the interaction of the *MYH9* mutation with other, not yet identified genetic and/or environmental factors [Heath et al. 2001; Kunishima et al., 2001b; Seri et al., 2003]. For this entity, the name *MYH9*-related disease (*MYH9*-RD) has been proposed [Balduini et al., 2002; Seri et al., 2003; Geddis and Kaushansky, 2004]. As a consequence, we have no means by which to predict in young patients with *MYH9* mutations the evolution of their illness, and in particular, to assess their risk of developing nephritis and CRF.

We present here the results of an analysis of the largest case series of patients with *MYH9*-RD so far collected, which demonstrates that the site of the *MYH9* mutation is indeed the main, and in some cases the only, determinant of the clinical picture of the disease.

PATIENTS AND METHODS

Patients

This study included all of the consecutive patients with *MYH9*-RD confirmed by molecular analysis diagnosed by our group from January 2001 to December 2006. Some patients had been previously reported (Table 1) [Seri et al., 2003; Pujol-Moix et al., 2004]. Mutational screening was performed as previously described [Seri et al., 2003]. MYH9 cDNA according to GenBank (RefSeq NM_002473.3) was used, with the A of the ATG translation initiation start site as nucleotide+1. The initiation codon is identified as codon 1. Ten previously reported patients (five families) had their genetic analysis performed by another group [Pujol-Moix et al., 2004]. All of the previously reported patients were followed-up after the first description by periodical clinical reevaluations, and their phenotypes have been updated for this study. Written informed consent was obtained in all cases. The study was approved by the Institutional Review Board of IRCCS Policlinico San Matteo Foundation, Pavia, Italy.

Phenotype Evaluation

Clinical features of *MYH9*-RD (macrothrombocytopenia, nephritis, CRF, sensorineural hearing loss, and cataracts) were carefully searched for in all enrolled patients, independently of the presence of a symptomatic disease. A patient was considered as evaluable for a given phenotype only whenever results of the specific examinations to investigate the phenotype (see below) were available. Enrolled patients underwent clinical reevaluations at least once a year. The patient's age included in the analysis was that at the time of the last clinical evaluation. In most patients, the automated and/or microscopic platelet count was repeated more than once, and the mean value was recorded. Patients have been defined as affected by *MYH9*-related nephritis when quantitative 24-hr proteinuria was more than 0.5 g in at least two consecutive examinations, in the absence of any other possible cause of proteinuria. CRF has been recorded for values of serum creatinine at least 1.5-fold higher than the upper reference value in at least two consecutive examinations, in the absence of any other cause of kidney damage. The presence of hearing loss was defined on the basis of the results of audiometric examination. Sensorineural deafness was recorded for a bone threshold average greater than 25 dB at 1000 Hz, 2000 Hz, and 4000 Hz. For infants the hearing function was examined by sensory evoked potentials. Presenile cataract was searched for by ophthalmological evaluation.

Statistical Analysis

Continuous variables were described as mean and standard deviation (SD) or as median and interquartile range (IQR), and categorical variables with counts and percent. We assessed the association of genotype (domain, type, and position of mutation) with the occurrence of noncongenital phenotypes (nephritis, CRF, sensorineural deafness, and presenile cataract) over time by means of event-free survival analysis. The patient's age was used to measure time. The follow-up was stopped at the date of diagnosis of the noncongenital defect or at the last available assessment of phenotype in the case of censoring. Kaplan Meier event-free

TABLE 1. **Results of Mutational Screening in the 108 Consecutive *MYH9*-RD Patients Enrolled for This Study***

NMMHC-IIA amino acid position	NMMHC-IIA domain	Exon	Amino acid change	Nucleotide change[a]	No. of patients (no. of families)		
					Total enrolled	Previously reported	Ref
93	MD	1	p.N93K	c.279C>G	1 (1)	1 (1)	A
96	MD	1	p.S96L	c.287C>T	2 (2)	None	–
702	MD	16	p.R702C	c.2104C>T	9 (8)	5 (5)	A
			p.R702H	c.2105G>A	7 (4)	4 (3)	A
718	MD	16	p.R718W	**c.2152C>T**	1 (1)	None	–
1066	TD	24	p.E1066_A1072del	c.3195_3215del	1 (1)	1 (1)	A
1155	TD	25	p.T1155A	**c.3463A>G**	1 (1)	None	–
			p.T1155I	c.3464C>T	2 (1)	2 (1)	A
1165	TD	26	p.R1165C	c.3493C>T	8 (3)	6 (2)	A,B
1424	TD	30	p.D1424H	c.4270G>C	19 (4)	15 (3)	A
			p.D1424N	c.4270G>A	5 (4)	1 (1)	A
			p.D1424Y	c.4270G>T	1 (1)	1 (1)	B
1447	TD	30	p.D1447V	**c.4340A>T**	4 (1)	None	–
1841	TD	38	p.E1841K	c.5521G>A	20 (7)	7 (2)	A,B
1925	TD	40	p.D1925TfsX23	**c.5773delG**	4 (1)	none	–
1933	TD	40	p.R1933X	c.5797C>T	13 (6)	6 (3)	A,B
			p.R1933EfsX15	**c.5797delC**	2 (1)	None	–
1941	TD	40	p.D1941MfsX7	c.5821delG[b]	5 (2)	None	–
1945	TD	40	p.E1945X	c.5833G>T	3 (1)	2 (1)	A
Totals					108 (50)	51 (24)	

*A total of 51 patients have been already reported [Seri et al., 2003; Pujol-Moix et al., 2004], while 57 cases are described for the first time in the present work. All of the previously reported patients were followed-up after the first description by periodical clinical reevaluations, and their phenotypes have been updated for this study. Novel mutations are shown in bold.

[a]A of the ATG translation initiation start site of the MYH9 cDNA in GenBank reference sequence NM_002473.3 is indicated as nucleotide +1.

[b]The c.5821delG mutation, which is named according to the current guidelines for the description of sequence variations (www.hgvs.org/mutnomen), was previously reported as 5818delG [Kunishima et al., 2003]. A = Seri et al. [2003]; B = Pujol-Moix et al. [2004]. MD, motor domain; TD, tail domain; Ref, reference.

survival was plotted, according to genetic characteristics. Rates per 100 person year and their Jacknife 95% confidence intervals (95%CI) were calculated. Cox regression was used to quantify the association of genotype and phenotype; hazard ratios (HR) and their 95%CIs were computed. The intra-familial aggregation was accounted for and Jacknife-Huber-White robust standard errors were used. Finally, the association of genotype with the degree of thrombocytopenia was assessed by a generalized estimating equations (GEE) population-averaged linear model (with exchangeable correlation), while accounting for familial aggregation. Mean differences (with respect to a reference category) and 95%CIs were computed to measure the effect of genotype on phenotype.

Stata 9.2 (Stata Corporation, College Station, TX) was used for computation. A two-sided P value <0.05 was considered statistically significant. Bonferroni correction was applied for post-hoc comparisons, resulting in a statistical significance if the P value was <0.017 in comparisons between types of mutation, and if the P value was <0.008 in comparisons between different positions.

RESULTS

Case Series and Mutational Analysis

A total of 108 consecutive patients belonging to 50 unrelated pedigrees with *MYH9*-RD were enrolled. Patients were 52 males and 56 females, and their mean age was 35 years (SD 10). In all cases the diagnosis was confirmed by mutational screening leading to the identification of 19 different, including five novel mutations of the *MYH9* gene (Table 1).

Of the novel mutations, each detected in only one family, three were missense alterations, p.R718W (c.2152C>T in exon 16), p.T1155A (c.3463A>G in exon 25), and p.D1447V (c.4340A>T in exon 30). The other two were single nucleotide deletions c.5773delG and c.5797delC, leading to prematurely truncated proteins p.D1925TfsX23 and p.R1933EfsX15, respectively. They are, as is the case for all *MYH9* frameshift or nonsense mutations reported in literature, localized within the last exon (exon 40) [Kunishima et al., 2001a, 2001b]. p.R718W and p.T1155A occurred as de novo mutations, being absent in patient's parents; the *MYH9*-RD phenotype appeared concurrently with the mutation in both families. The other three mutations cosegregated with the disease in the respective pedigrees. Of the three residues affected by novel missense mutations, threonine 1155 and aspartic acid 1447 were previously found to be mutated in *MYH9*-RD patients (p.T1155I and p.D1447G, respectively) [Seri et al., 2003; Schleinitz et al., 2006], whereas arginine 718 has been found to be altered for the first time. All novel missense mutations were undetectable in 100 normal individuals. Finally, the amino acid alignment of myosin homologs and orthologs showed conservation of the arginine 718, suggesting that it exerts a fundamental role in NMMHC-IIA structure and function.

We approached the question of genotype–phenotype correlations by grouping patients according to the position and/or type of the alteration of NMMHC-IIA protein. The *MYH9* mutations we have found affected 14 different amino acid positions of NMMHC-IIA (Table 1). In 16 families (32%), mutations affected a residue of the MD of NMMHC-IIA; in the remaining 34 pedigrees (68%), mutations altered the TD. All of the MD mutations were missense alterations, causing a single residue substitution in the globular head of the NMMHC-IIA. Within the TD, three different kinds of mutations were found: 1) missense mutations affecting the residues 1155, 1165, 1424, 1447, or 1841 (TD substitutions, TD-sub), 22 families (44%); 2) nonsense or frameshift mutations of the last exon, all resulting in the deletion of the last 13–36 residues of the C-terminus of NMMHC-IIA (TD C-terminal deletions, TD-Cdel), 11 families (22%); 3) an in-frame deletion of 21 nucleotides in exon 24 found in a single pedigree (2%) [Seri et al., 2003].

Of note, in 68% of families only four positions of NMMHC-IIA were affected: position 702 in the MD (12 families), and the positions 1424, 1841, and 1933 in the TD (9, 7, and 6 pedigrees,

respectively). A total of 74 patients carried mutations altering one of these four residues.

On the basis of these results, we performed three different analyses to search for genotype–phenotype correlations: 1) first, patients were grouped according to the NMMHC-IIA domain altered by mutation (MD mutations vs. TD mutations); 2) a further analysis was done by subdividing patients with TD mutations according to the type of protein alteration (MD mutations vs. TD-sub vs. TD-Cdel); and 3) a third analysis was performed by grouping patients according to the specific residue of NMMHC-IIA affected by mutation and comparing the four most frequently affected positions (position 702 vs. 1424 vs. 1841 vs. 1933).

Genotype–Phenotype Correlations

Nephritis and CRF. A total of 26 out of 93 evaluable patients (28%) developed the *MYH9*-related nephritis (mean age of evaluable cases, 33 years). The mean age at onset was 23 years (SD 9), and 77% of cases were diagnosed before the age of 30 years. The complication was found in 21 out of 44 evaluable pedigrees (48%). This picture resulted in an overall rate per 100 person year of 0.84 (95%CI 0.52–1.34).

Genotype–phenotype correlations with regard to nephritis are detailed in Table 2. Patients with mutations in the MD showed a greatly increased risk of developing this complication (rate per 100 person year = 4.00, 95%CI 2.89–5.47) with respect to cases with a TD mutation (0.47, 95%CI 0.25–0.94) ($P<0.001$). Of note, the event-free survival analysis showed that all of the patients with MD mutations are expected to develop nephritis before the age of 40 (Fig. 1A). In particular, mutations in position 702 of the MD showed a higher incidence compared to each of the TD positions 1424, 1841, and 1933 ($P<0.001$ for all the three comparisons) (Fig. 1B). Within the TD, the deletion at residue 1933 was associated with a lower risk of nephritis with respect to substitutions in positions 1424 and 1841 ($P<0.001$ in both cases). On the contrary, the incidence appeared similar when TD-sub and TD-Cdel subgroups were compared ($P=0.461$), thus suggesting that the better outcome is not a distinctive feature of the group of mutations in exon 40 leading to a truncated protein. Finally, the higher incidence observed for position 1424 compared to position 1841 did not result in statistical significance.

CRF was present in 19 out of the 100 patients who underwent the measurement of serum creatinine. Thus, 19 out of the 26 cases with nephritis (73%) had evolved to CRF at the time of evaluation. The mean age at onset was 26 years (SD 9). The complication was present in 15 out of 47 evaluable pedigrees (32%). The resulting overall rate per 100 person year was 0.55 (95%CI 0.33–0.94). All of the genotype–phenotype correlations observed for the variable nephritis were detected also when the variable CRF was examined, with the same levels of statistical significance (data not shown).

Sensorineural deafness. The hearing impairment was the most frequent nonhematological manifestation of *MYH9*-RD. Sensorineural deafness was present in 47 out of 78 evaluable patients (60%) and in 29 out of 41 families (71%). A hearing loss interfering with activities of daily living was reported by 43 out of 47 patients (91%) with altered audiometric examination. The mean age at onset was 31 years (SD 17). Differently from kidney involvement, the ages at onset were homogeneously distributed along the decades from first to sixth, since 33% of patients developed deafness before 20 years of age, 31% between 20 and 40, and 36% after 40. The resulting overall rate per 100 person year was 2.08 (95%CI 1.56–2.81).

Patients with MD mutations showed a considerably higher risk of developing sensorineural deafness than cases with a TD alteration ($P<0.001$) (Table 3). Of note, as for nephropathy, the event-free survival analysis demonstrated that all of the subjects with MD mutations are expected to develop the hearing impairment before the age of 40 years (Fig. 2A). The increased risk was observed also when MD mutations were compared separately with TDsub and TD-Cdel, which, on the contrary, did not show a significant difference between each other. The MD position 702 was associated with an increased risk compared to each of the TD positions 1424, 1841, and 1933 ($P=0.001$, <0.001, and <0.001, respectively). Within the TD, the deletion in position 1933 showed a significantly lower rate per 100 person year compared to substitutions in position 1424 ($P<0.001$), while positions 1424 and 1841 did not show any significant difference between them, as well as 1841 and 1933 (Table 3; Fig. 2B).

Presenile cataract. Presenile cataract was observed in 13 out of 82 evaluable cases (16%), and the mean age at onset was 23 years (SD 16). This complication was detected in seven out of 40 pedigrees (17%), with an overall rate per 100 person year of 0.48 (95%CI 0.19–1.71).

TABLE 2. **Correlations Between Genotype And Occurrence of Nephritis in 93 MYH9-RD Patients**[†]

	No. of patients, (families)	Affected patients, n (%)	Mean age at onset, years (SD)	Rate per 100 person year (95% CI)	HR (95% CI)	P value
NMMHC-IIA domain						**<0.001**
TD mutations	**75 (30)**	**13 (17)**	**26 (10)**	**0.47 (0.25–0.94)**	**1**	
MD mutations	**18 (14)**	**13 (72)**	**20 (8)**	**4.00 (2.89–5.47)**	**10.38 (4.68–23.04)**	
Type of mutation						**<0.001**
TD-Cdel	**25 (11)**	**3 (12)**	**26 (7)**	**0.31 (0.06–3.69)**	**1**	
TD-sub	**49 (29)**	**10 (20)**	**26 (11)**	**0.55 (0.27–1.21)**	**1.80 (0.38–8.56)**	**0.461**
MD mutations	**18 (14)**	**13 (72)**	**20 (8)**	**4.00 (2.89–5.47)**	**15.72 (3.42–72.31)***	**<0.001**
Position of mutation						**<0.001**
Position 702	**15 (11)**	**11 (73)**	**20 (6)**	**3.99 (2.76–5.64)**	**1**	
Position 1424	**22 (7)**	**7 (32)**	**29 (10)**	**1.06 (0.41–2.54)**	**0.17 (0.06–0.46)**	**<0.001**
Position 1841	**17 (6)**	**2 (12)**	**26 (8)**	**0.27 (0.06–1.85)**	**0.05 (0.01–0.24)****	**<0.001**
Position 1933	**13 (6)**	**0**	**–**	**0.00**	**n.e.*****	**<0.001**

[†]**Three different analysis were performed. Patients were first grouped according to the NMMHC-IIA domain altered by mutation. A further analysis was done by subdividing patients according to the type of TD alteration, substitution (TD-sub) or C-terminus deletion (TD-Cdel). A third analysis was performed by grouping patients according to the specific residue of NMMHC-IIA affected by mutation (position of mutation). Bonferroni correction for post-hoc comparisons within type of mutation significance if $P<0.017$; within position if $P<0.008$. The two patients with p.R1933EfsX15 mutation were not included in the "position 1933" subgroup, together with p.R1933X patients. However, also if they were included, all the reported results would be confirmed (data not shown).**

***$P<0.001$ vs. TD-sub.**

**** $P=0.118$ vs. position 1424.**

*****$P<0.001$ vs. position 1424 and position 1841. n.e. = not evaluable.**

No difference in the risk of developing cataracts was observed between MD and TD mutations. With regard to the four most frequently involved positions, a higher rate per 100 person year was calculated for position 1424 (Table 4). This figure was significantly higher compared to each of the other two TD positions 1841 and 1933 ($P = 0.003$ and <0.001), but not significantly different with respect to the MD position 702. Moreover, the risk associated with the 1933 position was significantly lower than that of each of the other three analyzed positions ($P<0.001$ for all the three comparisons).

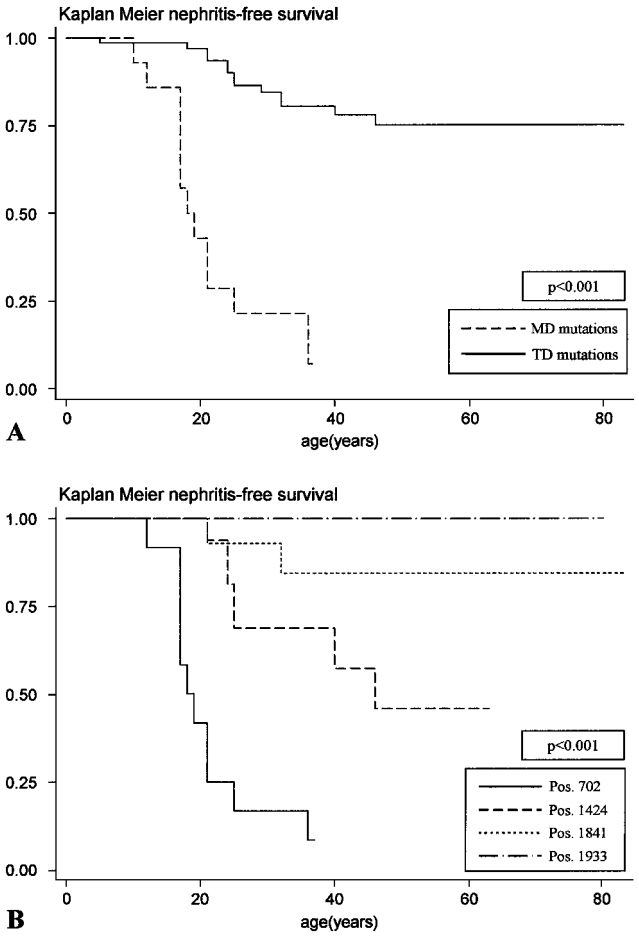

FIGURE 1. **Event-free survival analysis for the occurrence of nephritis. The association of genotype with the occurrence of nephritis was assessed over time by means of event-free survival analysis. Patients were grouped according to the domain (A) or the specific residue (B) of NMMHC-IIA affected by *MYH9* mutations. The patient's age was used to measure time. The follow-up was stopped at the date of diagnosis of the nephritis or at the last available assessment of phenotype in the case of censoring. MD, motor domain; TD, tail domain; Pos., position.**

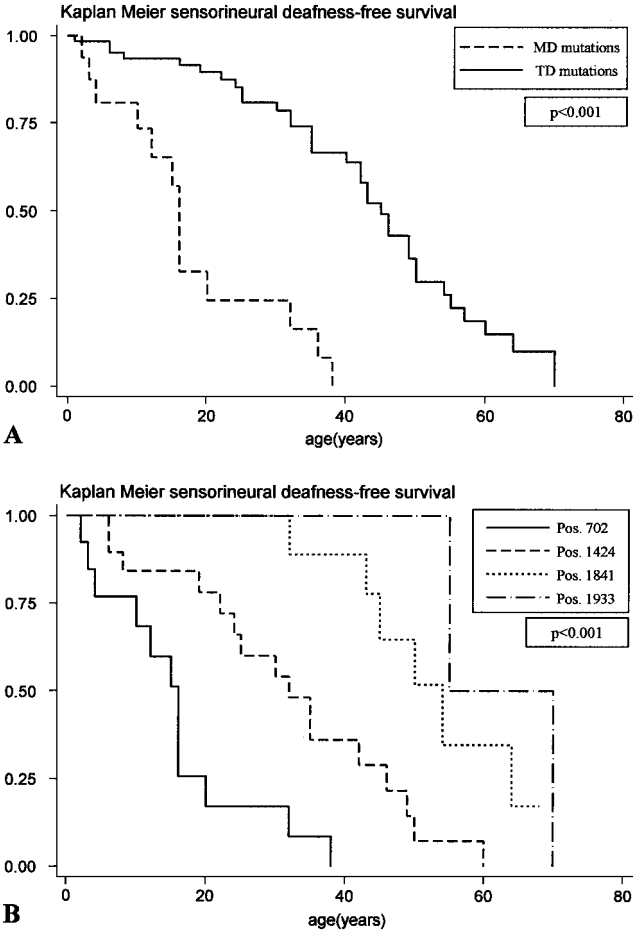

FIGURE 2. **Event-free survival analysis for the occurrence of sensorineural deafness. Patients were grouped according to the domain (A) or the specific residue (B) of NMMHC-IIA affected by *MYH9* mutations. MD, motor domain; TD, tail domain; Pos., position.**

TABLE 3. **Correlations Between Genotype and Occurrence of Sensorineural Deafness in 78 MYH9-RD Patients†**

	No. of patients, (families)	Affected patients, n (%)	Mean age at onset, years (SD)	Rate per 100 person year (95%CI)	HR (95%CI)	P value
NMMHC-IIA domain						<0.001
TD mutations	62 (28)	34 (55)	37 (17)	1.69 (1.19–2.50)	1	
MD mutations	16 (13)	13 (81)	17 (12)	5.35 (3.46–8.12)	6.88 (3.14–15.05)	
Type of mutation						<0.001
TD-Cdel	18 (9)	5 (28)	48 (20)	0.78 (0.36–2.25)	1	
TD-sub	43 (18)	28 (65)	36 (15)	2.03 (1.42–3.05)	3.55 (1.11–11.35)	0.033
MD mutations	16 (13)	13 (81)	17 (12)	5.35 (3.46–8.12)	20.90 (5.98–73.05)*	<0.001
Position of mutation						<0.001
Position 702	13 (10)	12 (92)	15 (11)	6.22 (3.78–9.80)	1	
Position 1424	20 (7)	16 (80)	30 (16)	2.89 (2.01–3.89)	0.25 (0.11–0.57)	0.001
Position 1841	14 (6)	6 (43)	48 (11)	1.09 (0.48–2.51)	0.06 (0.02–0.19)**	<0.001
Position 1933	7 (4)	2 (29)	62 (11)	0.71 (0.36–0.83)	0.02 (0.01–0.05)***	<0.001

†Bonferroni correction for post-hoc comparisons within type of mutation significance if $P < 0.017$; within position if $P < 0.008$.
*$P < 0.001$ vs. TD-sub.
**$P = 0.012$ vs. position 1424.
***$P < 0.001$ vs. position 1424 and $P = 0.034$ vs. position 1841.

Thrombocytopenia. Similarly to all macrothrombocytopenias with giant platelets, also in *MYH9*-RD the routine automated cell count overestimates the severity of thrombocytopenia, because cell counters fail to recognize very large platelets [Seri et al., 2003]. For this reason, the actual platelet count can be assessed only by manual counting upon phase contrast microscopy. In our case series, the platelet count measured by automated instruments in all 108 patients was 31×10^9/L (median, IQR 13–49). Microscopic platelet count was available for 60 cases, and the result was 68×10^9/L (median, IQR 37–93). In the same patient population, the median platelet count by cell counters was 16×10^9/L. Of note, in three patients, the microscopic platelet count was within the normal range (153, 159, and 178×10^9/L).

Genotype–phenotype correlations for thrombocytopenia are detailed in Table 5. Patients with a mutation in the MD had a lower microscopic platelet count than patients with a mutation in the TD ($P = 0.002$). Statistical significance was reached also when the MD position 702 was compared with each of the TD positions 1424, 1841, and 1933 ($P < 0.001$, 0.002, and 0.003, respectively), which, on the contrary, did not show any difference between each other.

Similar results were obtained when the automated platelet counts of all the 108 patients were considered (data not shown).

DISCUSSION

The clinical picture deriving from *MYH9* mutations is highly variable, with some patients presenting with only macrothrombocytopenia during their lifetime and some others developing during infancy or adult life the additional features of kidney failure, hearing loss, and/or presenile cataracts [Heath et al., 2001; Kunishima et al., 2001b; Seri et al., 2003; Dong et al., 2005]. Moreover, the degree of thrombocytopenia ranges from severe to mild, and a few subjects with normal platelet counts have been also reported [Noris et al., 1998; Seri et al., 2003]. The attempts that have been made to explain the differences in phenotypic expression on the basis of patients' genotypes have failed to demonstrate consistent correlations [Heath et al., 2001; Kunishima et al., 2001b; Seri et al., 2003], although they have suggested that non-hematological complications are more frequent in families with R702 mutations [Heath et al., 2001; Dong et al., 2005]. This led to the concept that the *MYH9*-RD phenotype results from the joint effect of *MYH9* mutations and yet unknown environmental and/or genetic factors, such as multiple gene products [Heath et al., 2001; Kunishima et al., 2001b; Balduini et al., 2002; Seri et al., 2003].

At present, it is therefore impossible to foresee in young patients the natural evolution of their illness, and this hampers not only genetic counseling and prognostic assessment, but also therapeutic approach. For instance, identification of patients at risk of developing CRF could allow close follow-up to be scheduled in order to discover the occurrence of kidney damage at its earliest phase and use drugs, such as ACE-inhibitors, that are effective in reducing proteinuria and slowing down worsening of renal function. Moreover, subjects at risk of developing defects of the inner ear, lens, or kidney should avoid the use of drugs that are potentially detrimental to these organs.

TABLE 4. **Correlations Between Genotype and Occurrence of Cataracts in 82 MYH9-RD Patients**[†]

	No. of patients, (families)	Affected patients, n (%)	Mean age at onset, years (SD)	Rate per 100 person year (95%CI)	HR (95%CI)	P value
NMMHC-IIA domain						0.581
TD mutations	67 (27)	11 (16)	23 (18)	0.45 (0.14–2.31)	1	
MD mutations	15 (13)	2 (13)	24 (3)	0.63 (0.15–4.84)	1.64 (0.28–9.54)	
Type of mutation						0.060
TD-Cdel	21 (9)	0	–	0.00	n.e.	<0.001
TD-sub	45 (17)	10 (22)	24 (17)	0.62 (0.18–3.72)	1	
MD mutations	15 (13)	2 (13)	24 (3)	0.63 (0.15–4.84)	1.30 (0.21–7.82)	0.777
Position of mutation						<0.001
Position 702	13 (11)	2 (15)	24 (3)	0.76 (0.19–5.48)	1	
Position 1424	19 (6)	8 (42)	20 (15)	1.61 (0.69–7.37)	1.40 (0.30–6.56)	0.666
Position 1841	16 (6)	2 (12)	44 (2)	0.28 (0.09–1.40)	0.24 (0.04–1.26)*	0.092
Position 1933	9 (4)	0	–	0.00	n.e.**	<0.001

[†]Bonferroni correction for post-hoc comparisons within type of mutation significance if $P < 0.017$; within position if $P < 0.008$.
* $P = 0.003$ vs. position 1424.
**$P < 0.001$ vs. position 1424 and position 1841. n.e., not evaluable.

TABLE 5. **Correlations Between Genotype and Platelet Count as Determined by Phase Contrast Microscopy in 60 MYH9-RD Patients**[†]

	No. of patients	Median platelet count (IQR), $\times 10^9$/L	Estimated difference (95% CI) (based on GEE model)	P value
NMMHC-IIA domain				0.002
TD mutations	48	80 (56–94)		
MD mutations	12	34 (25–39)	−19 (−31 to −7)	
Type of mutation				<0.001
TD-Cdel	11	90 (50–104)	–	
TD-sub	36	72 (56–94)	−1 (−34 to 32)	0.949
MD mutations	12	34 (25–39)	−46 (−78 to −15)*	0.004
Position of mutation				<0.001
Position 702	9	34 (25–37)	–	
Position 1424	12	65 (55–93)	43 (25 to 61)	<0.001
Position 1841	14	67 (24–82)	43 (16 to 70)**	0.002
Position 1933	6	88 (50–93)	46 (15 to 77)***	0.003

[†]Bonferroni correction for post-hoc comparisons within type of mutation significance if $P < 0.017$; within position if $P < 0.008$.
*$P < 0.001$ vs. TD-sub.
**$P = 0.989$ vs. position 1424.
***$P = 0.864$ vs. Position 1424 and $P = 0.876$ vs. position 1841.

The present study significantly improves our knowledge in this field, demonstrating in the largest case series collected so far that patients with mutations in the MD of NMMHC-IIA have a worse prognosis than subjects with TD alterations. Moreover, it allows the quantification of the risk of non-hematological manifestations in patients with mutations in four positions of NMMHC-IIA, which were responsible for the disease in 68% of our families and in 70% of pedigrees reported in the literature [Dong et al., 2005; Ma et al., 2006; Otsubo et al., 2006; Schleinitz et al., 2006].

Upon grouping together cases with mutations in the MD or TD, the Cox regression model for assessment of risk over time demonstrated that patients' prognosis is considerably different. In fact, all patients with MD mutations are expected to develop kidney impairment before the age of 40, while only 25% of subjects with TD mutations will present with this complication during their lifetimes. Similarly, all patients with MD mutations are predicted to suffer from sensorineural deafness before the age of 40, while at the same age, only 35% of cases with TD mutations will present with this phenotype. Alteration of the MD or TD strongly correlated also with the degree of thrombocytopenia, which was severe in subjects with MD mutations (median platelet count, 34×10^9/L), and mild in TD patients (80×10^9/L). This difference is clinically relevant, since platelet count associated with MD mutations exposes patients to the risk of severe bleeding, while that of TD mutations does not. Interestingly, the observation that three patients with TD changes had normal platelet counts confirms previous reports [Noris et al., 1998] and demonstrates that thrombocytopenia is not an absolute requirement for the diagnosis of *MYH9*-RD. Finally, cataract represents an exception to the observation of worse evolution associated with MD mutations, since its prevalence was similar in MD and TD alterations.

The analysis comparing mutations at the four most frequently affected residues of NMMHC-IIA further improves our prognostic ability for *MYH9*-RD. First, it statistically proved that patients with mutations at residue 702 of the MD present with more severe thrombocytopenia and a much higher risk of developing kidney and hearing defects than subjects with mutations in position 1424, 1841, or 1933 of the TD. Moreover, it allowed the identification of relevant prognostic differences also between the three most frequently affected positions of the TD. In fact, patients with the p.R1933X deletion showed no propensity to develop kidney impairment and cataracts and a later onset of deafness with respect to subjects with substitutions in positions 1424 or 1841. Within the TD, the mutations at residue 1424 were associated with a higher risk of developing cataracts and a trend for a higher incidence of sensorineural deafness. Finally, the p.E1841K substitution was associated with an intermediate or low risk profile for all the analyzed phenotypes.

In summary, two different clinical pictures of *MYH9*-RD clearly emerge. The most severe one, with very low platelet count, constant kidney damage and deafness before the age of 40 years, and a low risk of cataracts is always observed in patients with mutations affecting the arginine 702 (32% of our families), while the most favorable one, with only mild thrombocytopenia and hearing loss in the elderly, is a constant feature of subjects with p.R1933X (12% of families). The former or the latter clinical picture, or even intermediate forms, may occur in subjects with substitutions in positions 1424 or 1841 (32% of families). As already noted, the phenotype of *MYH9*-RD patients has been considered so far as the consequence of the interaction of *MYH9* mutations with the individual genetic backgrounds [Heath et al., 2001; Kunishima et al., 2001b; Seri et al., 2003]. Our results demonstrate that this is true only for some of the *MYH9* mutations. In fact, the consistently poor or good prognosis of patients with mutations in positions 702 and 1933, respectively, indicates that in these cases the effect on phenotype of factors other than NMMHC-IIA alterations is almost irrelevant. On the contrary, the variable expressivity of disease consequent to mutations at residues 1424 and 1841 suggests that in these cases other, yet unknown factors interact with *MYH9* defects and modulate their biological and clinical consequences.

The identification of definite genotype–phenotype correlations is also relevant to the debate of whether haploinsufficiency or dominant negative effect of the mutated allele is operative in *MYH9*-RD. The haploinsufficiency hypothesis has been suggested by the finding that both platelets and megakaryocytes from *MYH9*-RD patients present about 50% reduced expression of NMMHC-IIA with respect to healthy subjects at immunoblotting analysis [Deutsch et al, 2003; Pecci et al., 2005]. The dominant negative effect of mutated NMMHC-IIA has been supported by the observation that wild-type protein is sequestered together with the mutant molecule into Döhle-like inclusions of neutrophils [Kunishima et al., 2003; Pecci et al., 2005]. The dominant negative mechanism was indirectly supported also by murine models that showed that mice heterozygous for targeted disruption of Myh9 show a normal phenotype, thus indicating that haploinsufficiency does not reproduce the clinical picture of *MYH9*-RD [Matsushita et al., 2004]. Our observation that the site of *MYH9* mutations predicts both the severity of thrombocytopenia and the risk of non-hematological manifestations further supports the dominant negative mechanism, since haploinsufficiency is evidently not consistent with the specificity of the effect of the different NMMHC-IIA alterations on phenotype. In view of the different findings obtained in this field, we have to assume that despite the reduction of NMMHC-IIA expression observed in platelets and megakaryocytes, an amount of mutated NMMHC-IIA is present in these cells. Mutated protein, according to both the functional consequences of mutation on the actomyosin cycle and its ability to interact with the wild-type protein, could interfere to a different extent with the function of normal molecule.

The clinical severity induced by changes in position 702 can be explained by the fact that arginine 702 is a highly-conserved residue in the "SH1-SH2" helix, which plays a key role in the transition between different conformational states of the globular head during the actomyosin cycle [Houdusse et al., 1999, 2000]. Both p.R702C and p.R702H mutations have been predicted to destabilize the secondary structure of the "SH1-SH2" helix [Seri et al., 2000; Heath et al., 2001], and the p.R702C NMMHC-IIA showed a striking reduction of in vitro ATPase activity and a severely impaired ability of moving actin [Hu et al., 2002]. Since NMMHC-IIA mutated in position 702 preserves a normal TD, it is likely that in vivo it copolymerizes with wild-type NMMHC-IIA and severely affects the function of the resulting filaments. On the contrary, mutations in positions 1424, 1841, and 1933 have been all predicted to hamper the NMMHC-IIA assembly into filaments by impairing the dimerization of NMMHC-IIAs in coiled-coil structures and/or perturbing the latero-lateral associations of NMMHC-IIA dimers. As a consequence, the capacity of these mutant NMMHC-IIAs to copolymerize with wild-type molecules into filaments is reduced and therefore their "poisoning effect" on the normal protein is less severe. This hypothesis is supported by the previous observation that the p.R1933X deletion, which correlates with the mildest phenotype, is completely unable to form in vitro ordered paracrystalline structures typical of

NMMHC-IIA assembly [Franke et al., 2005], and therefore it is potentially unable to interact with the wild-type molecule and exert its poisoning effect. On the contrary, the p.D1424N protein, which is associated with a less favorable phenotype, was found to retain the ability to form paracrystalline assemblies with altered morphology when mixed with wild-type molecule [Franke et al., 2005]. Obviously, further experimental work is required to confirm or deny these speculations.

In conclusion, we identified statistically significant genotype–-phenotype correlations in *MYH9*-RD patients that define their prognosis and have practical consequences on their clinical management. Additional investigation is required to also obtain this kind of information for the genotypes, whose prognosis is still uncertain because of either the low number of investigated subjects or the intervention of environmental factors.

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