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Original Article

A Novel Molecular Indicator for Inhibitor Development in Haemophilia A

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Aim: Inhibitor development (ID) risk in the patients with haemophilia A(HA) having missense mutations has been reported to be 3-10% in other studies. We investigated the association between ID risk and various features of missense mutations including aminoacid group change caused by.

Methods: Missense mutations in the F8 gene, patients' clinical findings including severity of HA and ID status were obtained from the F8 gene variant database(http://www.factorviii-db.org/). Twenty aminoacids are classified into groups according to their side chains. All information about each mutation and whether the mutation caused aminoacid group change were recorded. Additionally, localisation (at which domain) of the changed aminoacid in the F8 protein was recorded. In this study, we used CADD, REVEL, M-CAP, and DANN scores to find a significant cut-off value indicating ID.

Results: We found three features that could be predicted to ID in mild HA: First, Among mild HA patients, 7.9% (n=70/883) of patients with mutations causing no aminoacid group changes showed ID, this rate was only 2.9% in patients with mutations leading to aminoacid group changes. Second, patients with mutations causing no aminoacid group changes effecting A2, A3 and C2 domains, ID risk was found to be higher than the patients with mutations leading to aminoacid group changes. Third, CADD and REVEL scores have been found to be associated with ID.

Conclusion: In mild haemophilia A patients, the ability of aminoacid group changes of missense mutations, and CADD and REVEL scores could be suggested to use for predicting ID risk.

Keywords: Haemophilia A; Inhibitor; F8 gene; Mutation; Missense; Interpretation

Introduction

The development of neutralizing antibodies, which is called inhibitor development, against factor VIII (FVIII) is a serious complication of the early stages of replacement therapy in haemophilia A (HA). The overall incidence of inhibitor development is 20-30% [1]. The mechanisms underlying inhibitor devlopment is very complex and is not yet fully understood. Risk factors for inhibitor development that allow risk stratification are classified simply into two groups: modifiable and unmodifiable. The most important unmodifiable risk factors are the type of the causative mutation in F8 gene and the clinical severity[2–4]. Major

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modifiable risk factors are age at first treatment and the source of FVIII concentrate [5, 6].

There have been a number of studies investigating complex pathophysiological mechanisms leading to the development of FVIII inhibitors. Relationships between type of F8 mutation and risk of inhibitor formation have been extensively discussed in recent years. In 1995, Schwaab et al. reported that cumulative incidences of inhibitor development in severe HA patients carrying large deletions, nonsense mutations, and intron 22 inversions were 35.7%, 38.4% and 34.4% respectively whereas in the patients with missense F8 mutations this risk was 4.3% [2]. Inhibitor development risk for missense mutations has been reported to be 3 to 10% in several other studies [7, 8].

The position and type of subtitution of missense mutations may influence the risk of inhibitor development. The INSIGHT study analysed the association between F8 mutation and inhibitor development in 1112 patients with non-severe HA. Among a total of 214 different F8 missense mutations, 19 were reported to be associated with inhibitor development (p.Leu412Phe, p.Arg531Cys, p.Arg593Cys, p.Asn618Ser, p.Pro1761Gln, p.Phe1775Val, p.Arg1781Gly, p.Pro1854Leu, p.Arg1997Trp, p.Asp2074Gly, p.Phe2101Cys, p.Tyr2105Cys, p.Arg2150His, p.Arg2159Cys, p.Glu2228Asp, p.Trp2229Cys, p.Val2232Ala, p.His2309Asp, p.Ter2333Cys) [9]. It is still not clear why those missense mutations have the higher risk for the development of inhibitor.

In this study, we investigated the association between inhibitor development risk and various features of missense mutations including amino acid group change caused by.

Materials and Methods

Missense mutations in the F8 gene, patients' clinical findings including severity of HA and inhibitor development status were obtained from the F8 gene variant database (http://www.factorviii-db.org/) by European Association for Haemophilia and Allied Disorders (EAHAD). Cases with mutation (19 mutations) found to be associated with inhibitor development in the INSIGHT study were excluded [9].

Twenty aminoacids which make up structural proteins are classified into two major groups according to their side chains: nonpolar hydrophobic and polar. The polar group is then subclassified into 3 subgroups: basic, asidic and polar uncharged (Table 1) . All information about each mutation and whether the mutation caused amino acid group change were recorded. Additionally, localisation (at which domain) of the changed amino acid in the F8 protein was recorded.

In genetic era, in silico protein modelling programs are used to interpret the effects of a mutation at protein level . Almost all such programs give a probability scoring changing benign to pathogenic for the effect of a certain mutation. Each have unique alghorythm and cut-off value. In this study, we used CADD, REVEL, M-CAP, and DANN scores to find a significant cut-off value and a spesific marker indicating inhibitor development [10–13].

Statistical Analysis

Statistical package IBM SPSS version 25 (IBM Corp., Armonk, NY, USA) was used to analyze the data. The Chi-squared test was used to compare differences in the categorical data between groups and $p<0.05$ was regarded as statistically significant. Two-sample independent t-test was used for the numeric data and then to define a cut-off value ROC analysis was performed in data found statistically significant. All hypothesis tests were carried out at 0.05 significance level.

Results

All recorded cases (Until May 2019) were screened in the F8 gene variation database (http://www.factorviii-db.org/) and 3248 different cases with 954 different missense mutations and having necessary information for the study were recruited. The missense mutations recruited were in 607 different points in the cDNA of the F8 gene. Of 3248 cases 3078 had information about clinical severity of the disease and 2251 had information about inhibitor development. When the cases were evaluated according to their clinical severity, 1717 cases with missense mutation (55.8%) had mild HA, 639 (20.8%) moderate, and 722 (23,5%) severe HA. Thus, Of all 3078 cases 76.5% (n=2356) were mild or moderate HA. There were 2251 cases having information about their inhibitor status. Among these, 157 (7%) cases were recorded to be positive for inhibitor development against FVIII protein. In 2207 cases information about both clinical phenotype and inhibitor information was present and of these, 153 (%6.9) had been reported to be inhibitor positive. Using 4 groups classification (including subgroups of amino acids) (Table 1), evaluation according to whether substitution caused changes in the aminoacid class or not showed that mutations causing no amino acid group changes were more associated with inhibitor development ($p=0.012$). Inhibitor development rate was 8.9% (n=67/755) in cases with mutations resulting in no changes in amino acid group, while it was 6.0% (n=90/1496)in cases with mutations causing amino acid group changes. Difference between these two groups was more significant when

Table 1. Aminoacid classification according to their side chains.	
Amino acid class	**Amino acids**
Class 1: Nonpolar Hydrophobic	Ala, Val, Leu, Ile, Met, Pro, Phe
Class 2a: Polar uncharged	Ser, Tyr, Asn, Gln, Cys, Thr, His, Gly
Class 2b: Acidic	Asp, Glu
Class 2c: Basic	Lys, Arg

the cases differed between clinical severity; in mild HA 9.8% (n=44/451) ofpatients with mutations causing no amino acid group changes had inhibitors while only 4.8% (n=34/709) of patients with mutations causing amino acid group changes had inhibitors (p=0.001). In the moderate and severe HA patients, there was no significant difference between these two groups (Table 2).

Using 2 groups classification, evaluation of missense mutations, according to whether substitution caused changes in the aminoacid class or not, showed similarly that mutations causing no amino acid changes were more associated with inhibitor development in mild HA cases than the mutations resulting in amino acid changes (p=0.001); among mild HA patients, 7.9% (n=70/883) of patients with mutations causing no amino acid group changes showed inhibitor development, this rate was only 2.9% in patients with mutations leading to amino acid group changes. In moderate and severe HA, no statistical difference was detected between the grup, similar to 4 group classification (Table 2).

In this study associations between affected domains and inhibitor development were also investigated. There was no inhibitor development in patients having mutations effecting a1, a2, a3 and SP domains of the protein. The patients with mutations affecting C1 (11.4%) and C2 (10.8%) domains had the highest risk. The rates were 7.3%, 7.2% ,and 3.1% in patients with mutations effecting A2, A3 and A1 domains respectively.

If we combine the information in this study including clinical severity and aminoacid group changes, associations between affected domains and inhibitor developments were reevaluated. Using 4 group classification, in mild HA patients with mutations causing no amino acid group changes effecting A2, A3 and C2 domains, inhibitor development risk was found to be higher than the patients with mutations leading to amino acid group changes; inhibitor devolopment rates were 10.4% (n=18/173) versus 3.7% (n=7/188) for A2 domain (p=0.012), 7.6% (n=7/92) versus 1.4% (n=2/142) for A3 domain (p=0.016) and 20.8% (n=11/53) versus 7.3% (n=7/96) for C2 domain (p=0.016). (Table 3)

To find a significant cut-off value and a spesific marker indicating inhibitor development, we used CADD, REVEL, M-CAP, and DANN scores dividing the cases according to the clinical phenotype. There were no significant association between M-CAP or DANN scores and inhibitor development. However, in mild/moderate HA cases, CADD and REVEL scores have been found to be associated with inhibitor development. To establish a cut-off value, which indicates inhibitor development, ROC analysis has been applied. For CADD and REVEL scores, area under the curve was found to be significant in mild/moderate HA cases (Table 4) (Figure 1).

Discussion

In this study, for the first time in the literature, we showed that, in mild HA, there could be significant relationship between inhibitor development and whether mutation causes amino acid group change.

As it is well known, the degree of severity of the disease represents an important factor for the occurrence of inhibitor. In severe haemophilia, factors effecting inhibitor development are well known. These include familial predisposition, mutation type, human leucocyte antigen class II polymorphism, immunological factors, and environmental factors such as surgery and trauma [14–16]. In this respect, the type of mutation is the most important genetic factor. The treatment choices of the patients are made by using these factors.

It has been reported that there is approximately 3-10% risk of inhibitor development in mild haemophilia cases. Most of mild HA cases with inhibitors have missense mutations. However no predictive data related to features of the mutations in the F8 gene have been reported in terms of inhibitor development in these cases [17]. In this study, using information obtained from the F8 variant database, the risk of inhibitor development in mild haemophilia A cases was determined to be approximately 7%.

Risk factors for inhibitor development in severe haemophilia are well known, while in mild haemophilia, these factors are not clear. Although the family history of

Table 2. Relationship between inhibitor development and changes in amino acid class due to the missense mutation. In this table, statistics in four group classification indicates changing between nonpolar hydrophobic, basic, acidic and polar uncharged amino acids and statistics in two group classification indicates changing between nonpolar hydrophobic and polar amino acids (Table 1).

All patients (n=2251)		Changes in amino acid class (Four groups classification)		P value
		No	Yes	
Inhibitor development	No	688 (91.1%)	1406 (94%)	0,012
	Yes	67 (8.9%%)	90 (6.0%)	
In mild cases (n=1160)		Changes in aminoacid class (Four groups classification)		P value
		No	Yes	
Inhibitor development	No	407 (90.2%)	675 (95.2%)	0,001
	Yes	44 (9.8%)	34 (4.8%)	
In mild cases (n=1160)		Changes in aminoacid class (Two groups classification)		P value
		No	Yes	
Inhibitor development	No	813 (92.1%)	269 (97.1%)	0,001
	Yes	70 (7.9%)	8 (2.9%)	
In moderate cases (n=496)		Changes in aminoacid class (Four groups classification)		P value
		No	Yes	
Inhibitor development	No	123 (96.1%)	345 (93.8%)	0,322
	Yes	5 (3.9%)	23 (6.3%)	
In moderate cases (n=496)		Changes in aminoacid class (Two groups classification)		P value
		No	Yes	
Inhibitor development	No	326 (94.8%)	142 (93.4%)	0,549
	Yes	18 (5.2%)	10 (6.6%)	
In severe cases (n=551)		Changes in aminoacid class (Four groups classification)		P value
		No	Yes	
Inhibitor development	No	135 (89.4%)	369 (92.3%)	0,286
	Yes	16 (10.6%)	31 (7.8%)	
In severe cases (n=551)		Changes in aminoacid class (Two groups classification)		P value
		No	Yes	
Inhibitor development	No	352 (91.9%)	152 (90.5%)	0,322
	Yes	31 (8.1%)	16 (9.5%)	

Table 3. Relationship between inhibitor development and changes in aminoacid class due to the missense mutation in spesific domains (in this table, A2, A3 and C2 domains are shown)

Domain A2		**Changes in aminoacid class (Four groups classification)**		
In mild cases (n=361)		No	Yes	P value
Inhibitor development	No	155 (89.6%)	181 (96.3%)	0,012
	Yes	18 (10.4%)	7 (3.7%)	
In moderate cases (n=98)		No	Yes	P value
Inhibitor development	No	31 (93.9%)	62 (95.4%)	0,759
	Yes	2 (6.1%)	3 (4.6%)	
In severe cases (n=112)		No	Yes	P value
Inhibitor development	No	38 (88.4%)	62 (89.9%)	0,805
	Yes	5 (11.6%)	7 (10.1%)	
Domain A3		**Changes in aminoacid class (Four groups classification)**		
In mild cases (n=234)		No	Yes	P value
Inhibitor development	No	85 (92.4%)	140 (98.6%)	0,016
	Yes	7 (7.6%)	2 (1.4%)	
In moderate cases (n=110)		No	Yes	P value
Inhibitor development	No	32 (97.0%)	74 (96.1%)	0,824
	Yes	1 (3%)	3 (3.9%)	
In severe cases (n=101)		No	Yes	P value
Inhibitor development	No	16 (84.2%)	67 (81.7%)	0,797
	Yes	3 (15.8%)	15 (18.3%)	
Domain C2		**Changes in aminoacid class (Four groups classification)**		
In mild cases (n=149)		No	Yes	P value

Table 3. contiuned

Inhibitor development	No	42 (79.2%)	89 (92.7%)	0,016
	Yes	11 (20.8%)	7 (7.3%)	
In moderate cases (n=62)		No	Yes	P value
Inhibitor development	No	5 (83.3%)	49 (87.5%)	0,772
	Yes	1 (16.7%)	7 (12.5%)	
In severe cases (n=64)		No	Yes	P value
Inhibitor development	No	8 (100%)	52 (92.9%)	0,435
	Yes	0 (0%)	4 (6.3%)	

Table 4. A) The comparison between interpretation scores and inhibitor development and B) Cut-off values of interpretation scores and their sensitivity/specivity in the patients with mild/moderate HA.

(A) Scores	**Inhibitor development**	**N**	**Mean**	**Std. Deviation**	**P value**
CADD_phred	No	1550	27.87	±5.23	0.000
	Yes	106	29.80	±4.05	
CADD_raw	No	1550	5.74	±1.51	0.000
	Yes	106	6.41	±1.15	
REVEL_score	No	1550	0.84	±0.11	0.000
	Yes	106	0.89	±0.08	

(B) Scores	**Susceptible to inhibitor development if greater than or equal to**	**Sensitivity**	**Specivity**	**LR+**	**LR-**	**Area under the curve**	**P value**
CADD_phred	28,05	0,65	0,60	1,599	0,585	0,634	0,000
CADD_raw	6,11	0,65	0,61	1,647	0,573	0,640	0,000
REVEL_score	0,87	0,65	0,62	1,681	0,566	0,642	0,000

LR+: Positive likelihood ratio, LR-: Negative likelihood ratio.

inhibitor development and treatment-related factors have been reported as the risk factors, the specific mutation features have not been investigated detailed [18]. Certain F8 gene missense mutations contribute to the development of inhibitors in patients with mild haemophilia, sometimes up to the level observed patients with severe disease [7, 14]. This association with F8 mutations was first demonstrated in a cohort study by Eckhardt et al (2009). In that study conducting in 128 patients with mild HA and 10 patients with moderate HA, of ten patients who developed inhibitors, eight carried the Arg593Cys mutation [16]. Eckhardt et al (2013), further investigated the results of the INSIGHT study, a registry involving 34 haemophilia treatment centres acrossing 11 countries. In their study among a total of 214 different F8 missense mutations, 19 were found to be associated with inhibitor development (The INSIGHT

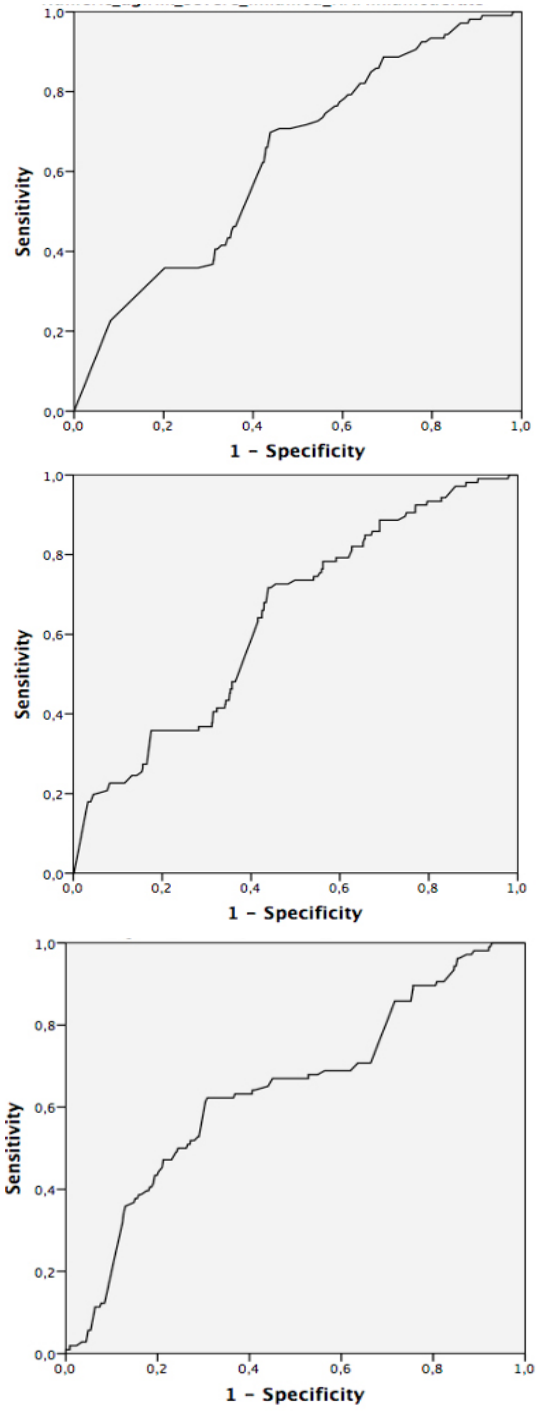

Figure 1. ROC analysis of A) CADD_phred_score B) CADD_raw_score C) REVEL_score.

Study) [9]. However, the link between different features of the missense mutations and inhibitor development risk has not been studied.

In our study, we detected 2 features that can be used to predict inhibitor risk development in mild haemophilia A cases caused by missense mutation. First, if the missense mutation found in mild HA patient does not cause amino acid group change, inhibitor development risk increases. Second, missense mutations causing no amino acid group change and effecting A2, A3 and C2 domains of F8 protein lead to a statistically significant higher risk of inhibitor development.

A number of previous studies confirmed the relationships between 19 misense mutations reported in the INSIGHT study and inhibitor development . In the study by Kempton et al (2012), 3 different missense mutations (R593C, R2150H, and N1922S) were found to causative for inhibitor development in their cohort including 18 mild/moderate haemophilia A patients. Two (R593C and R2150H) of these three mutations were also reported in the INSIGHT study, while the mutation N1922S was not. We consider that the N1922S mutation does not cause amino acid group change and therefore it could be a factor for inhibitor development [19]. Iioka et al reported a mild HA patient developing high titers of inhibitors. This patient had a missense mutation, c.3780C>G (p.D1241E), and required long term treatment course. They argued that the high titers of inhibitor development was due to the long treatment course. Regarding our study results we consider that in Iioka et al's patient, inhibitor development could be resulted from the mutation (c.3780C>G) that does not cause amino acid group change [20].

In this study, we also investigated predictive cut-of values of several mutation patogenicity scoring systems for inhibitor development risk. It has been considered that that CADD and REVEL scores can be used for this purpose (Table 4).

Conclusion

In mild haemophilia a patients , the ability of amino acid group changes of missense mutations, and CADD and REVEL scores could be suggested to use for predicting inhibitor development risk. Additionally missense mutations causing no amino acid group change and involving A2, A3 and C2 domains of the FVIII protein lead to a highest risk of inhibitor develpment. The weak point of our study is that no information has been provided about the treatment regimen of patients. It also depends on retrospective statistical analysis. Furher prospective studies including treatment regimens are needed to prove the hypoteses put forward in this study.

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