Molecular genetic and population studies of monogenic and recessive diseases

Thesis of Ph.D. dissertation

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INTRODUCTION:

Results of molecular genetics have great importance in the investigations, diagnostics and therapy of diseases. They provide help in establishing the diagnosis in cases, where clinical symptoms and other labor results do not refer unambigously to the genetic disease. They can help in distinguishing the hereditery form of the disease from the aquired form. Presymptomatic diagnosis is particularly important, because in some cases it is possible to prevent the developement of the disease with the targeted therapy, started in time. Another aim of DNA-diagnostics might be the finding of connections between mutation and course of disease. Identification of different mutations can give the possibility for developement of optimal therapy and lifestyle.

These standpoints are important in the diagnostics and prevention of mainly in the mild or moderate (but more frequent) disorders. Consequently, population studies can have a main role in the clearing up of the genetic background in certain diseases, improvement of specific therapies developed for individuals or groups. Testing of different mutations in different groups of patients can help in understanding the mechanism of formation of mutations, mapping of the origin of mutations, optimalisation of diagnostic protocols.

In cases of severe, hereditery diseases, where there is difficoult or no therapy, affected family-members offen claim for prenatal diagnosis. Modern genetic councelling is based on the exact determination of genetic status of an embryo Identifying of the healthy, but mutation-carrier family-members is especially important. Gene therapy, the causal therapy of severe hereditery diseases,is also based on the knowledge of the disease-gene and its mutations.

In the current work, I illustrate the above applications of molecular genetics by using four examples of monogenic, recessive diseases: juvenile haemochromatosis (JH), haemophilia A (HA) and haemophilia B (HB), and one type of hereditery non-syndromatic recessive deafnesses.

AIMS OF THE STUDY:

1.: In case of juvenile haemochromatosis, our aim was to prove the symptom-based probable diagnosis by molecular genetic investiagtions (direct sequence analysis of haemojuvelin (*HJV*) and hepcidin (*HAMP*) genes) in a male patient, who has died in his late twenties., and finding out the exact genetic background of the other members of the family. In case of the two symptom and complaint-free brothers of the patient we woud like to diagnose the possible presymptomatic status. The result of the genetic test might give new informations on understanding the function of the protein, and might help in finding answer for the especially fast progress and fatal outcome of this JH case.

2.: The aim of the haemophiliac molecular genetic program was to establish a diagnostic strategy, which optimally combines accurate and labor-intensive methods based on direct mutation detection with faster, but in some cases not absolutely informative indirect methods based on investigation of polimorphic markers, and is suitable for identifying women, who have no symptoms, but carry defected factor VIII or IX (carrier detection), and in cases of carriers, for prenatal diagnosis in the first trimester of pregnancy. Testing large number of HA and HB families, we wanted to check the applicability of the newly develeoped algorithm. Other aim was the determination of the mutation spectrum of the patients, and the heterozygosity rate of each examined polymorph markers in the Hungarian population and examination of the possible linkage disequilibrium between the markers, and compairing our results with the international data. The identified mutations can help in examining genotype-phenotype correlations, and in understanding structural characteristics.

3.: The allele frequencies for the two common disease causing mutations (c.35delG and c.167delT) of *GJB2* gene in the most common form of non-syndromatic recessive deafness cases were not previously investigated in the begining of our study in Ashkenazi and Romani populations in Europe.The aim of our work was to determine the allele frequencies of these mutations in randoomly selected groups of the Hungarian Ashkenazi and Romani populations, and to compare the data to the results of healthy control Caucasian population, First, we would like to find out, if the Hungarian Ashkenazi population is closer to the Hungarian control or to other Ahkenazi populations in respect of allele frequency of c.167delT mutation, and if there is any difference in the frequency of c.35delG mutation in the Romani and control Caucasian population.

METHODS

Subjects

- Juvenile haemochromatosis: 25-year old, healthy, moderate smoker and alcohol consumer manual worker man with negative family anamnesis. Beside the patient, molecular genetic investigation of the mother and two brothers without any complaint and symptoms were performed.
- Haemophilia A and B: 167 HA and 27 HB affected unrelated families and 38 independent severe HA patients participated in haemophilia genetic advising.
- Hereditery non-syndromatic recessive deafness: The allele frequencies of the two common mutations of the *GJB2* gene was determined in three groups. Control group consisted of 163 first-time blood donors. 346 unrelated individuals were investigated in the Romani population, Askneazi group consisted of 186 randoomly selected, unrelated individuals from Budapest. All three groups were representetive for the corresponding population.

Applied methods:

- DNA isolation: from anticoagulated peripheral blood samples and from chorionic villi samples (CVS) by standard ”salting out” procedure or by Puregene (Gentra) DNA Isolation Kit.
- Point mutation detection by PCR-RFLP method: for *HJV* gene G66X point mutation, *FVIII* gene *Bcl*I, *FIX* gene *Hha*I, *Taq*I, *Xmn*I, polimorphism, for *GJB2* gene c.35delG és c.167delT point mutations and for verifying newly identified missense (amino-acid change) mutations in *FVIII* and *FIX* genes.
- Microsatellite analyses for the *FVIII* gene IVS13CA and p39CA repeat-number identification: PCR amplification with fluorescent or radioactively labeled primers followed by polyacrylamide gel or capillary electrophoresis.
- Southern blot analysis: for *FVIII* gene intron 22 inversion detection.
- Long-distance PCR (LD-PCR) for *FVIII* gene intron 22 inversion detection.
- PCR: for *FVIII* intron 1 inversion and *FIX* DdeI polimorphism detection.
- Sequencing (in case of *HJV*, *FVIII* és *FIX* genes): dideoxi chain-termination method with capillary electrophoresis.

RESULTS:

1. By molecular genetic investigtion, we unambiguously confirmed the diagnosis based on phenotypic data in case of a patient, who died in a severe, rare disorder, juvenile haemochromatosis.

2. We identified a new point mutation in the hemojuvelin gene that causes the change of glycine in the 66. position of the protein to a stop codon (G66X), This mutation provides new informations on possible functions of the predicted alternative protein products, due to its special loacation. We confirmed the results by PCR-RFLP method, designed direct for this mutation.

3. By detecting the newly identified mutation, we diagnosed the hetereozygous status in the mother and two brothers of the patient, so in case of them the phenotypic appearance of the disease is not feasible.

4. We established a complex algorithm based on direct mutation detection (inton 22. and intron 1. inversion detection in *FVIII* gene, and direct sequencing of *FVIII* és *FIX* genes) and polimorph marker testing, (combination of IVS18 PCR-*Bcl*I-RFLP, IVS13CA and extragenic p39CA in case of *FVIII* gene and combination of *Hha*I, *Taq*I, *Xmn*I, and *Dde*I markers in case of *FIX* gene) which is a reliable and cost effective method in the carrier and prenatal diagnostics of Hungarian families with haemophilia.

5. We determined the heterozygoutic valeues of the examined polimorfic markers linked to the two genes in the Hungarian population (In case of *FVIII* gene: IVS18 PCR-*Bcl*I-RFLP: 42%, IVS13CA: 61%; p39CA: 82%, in case of *FIX* gene RFLP markers: *Hha*I 73%, *Taq*I 36%, *Xmn*I 23%, *Dde*I 21%). We concluded, that in haemophilia A examined markers are not linked to each ather in the Hungarian population.

6. By investigating 24 severe HA family, we identified 14 novel mutations (4 missense, 4 nonsense, 5 small insertions and deletions, one splice-site mutation). The patterns of the identified mutation types (nucleotide changes, ins/del, splice) argued with published data. We proved the disease-causing status of the mutations applying international recommendations.

7. Several carrier and prenatal tests were was performed by direct mutation detection and polimorph marker testing. Carrier status was excluded in 83, and confirmed in 46 cases among potentially HA-carrier women, by investigating 167 haemophilia A families. Mutation was detected or at least one infromative polimorf marker was identified in 188 HA-carriers, preatatal diagnostics was performed in case of 26 male fetuses.

8. Carrier and prenatal diagnostics was performed in 27 haemophilia B families, primarily by sequence analysis of the *FIX* gene. We identified three novel disease-causing mutations.

9. In three populations with different origin from Hungary we determined the allele-frequencies of the two most common point mutations (c.35delG and c.167delT) of *GJB2* gene, which encodes for connexin 26 protein and is in the background of hereditery non-syndromatic deafness.

10. The 0,6% allele frequency (1:80 carrier frequency) for c.35delG mutation in the control Caucasian population is similar to results based on different populations in Central-Europe. In case of c.167delT mutation, the 2,4% allele frequency in our Ashkenazi group is similar to other groups of the same origin but from different locations.

11. We analized the occurance of c.35delG mutation in the Romani population for the first time. We detected an allele-frequency of 0,4% in the healthy Romani group, which corresponds with the result of the control Caucasian population.

12. Our findings confirm the valeue of performing the c.35delG mutation detection in case of the patients and family members in suspect of hereditery non-syndromatic deafness also in Hungary. The adopted (for c.35delG mutation) and our new (for c.167delT) molecular genetic methods based on mutation detection can provide exact diagnosis in the differential diagnostics of hearing disorders

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CONCLUSIONS:

Conclusions from the molecular genetic examinations of *HJV* gene

Our results agree with the autosomal recessive heredity of juvenile haemochromatosis. By detecting the hetereozygous status we were able to give definite diagnosis to the two brothers of the patient, In case of a homozygous mutant status of the third brother the amount of the accumulated iron can be decreased and so the appearance of the severe phenotype prevented. The newly identified G66X mutation blocks only the formation of the full-length, 426 basepair-long protein theoretically. From the specific position of this mutation we can conclude, that the hypotetical alernative proteins do not have any role in the iron homeostasis.

Conclusions from the molecular genetic examinations of haemophilia A and B

The established complex algorithm based on direct mutation detection and polimorph marker testing is a reliable method in the carrier and prenatal diagnostics of Hungarian families with haemophilia. By direct sequence analysis we can perform carrier and prenatal tests in families, where investigations using polimorph markers do not give definite answers. Heterozygosity rate of the examined polymorph markers and mutation spectrum of the patients, are in good correlation with international data.

Conclusions from the population examinations of *GJB2* gene mutations

The allele frequency for c.35delG mutation in the control Caucasian population is similar to results based on different populations in Central-Europe. We did not find any c.35delG carrier in the examined Ashkenazi group. Our findings further confirm the founder effect of this mutation. We analized the occurance of c.35delG mutation in the Romani population for the first time. The 0,4% allele-frequency is in good correlation with the result of the control population. We can conclude, that the similar data come from the blending of populations during migration and after settlement. Clinical and auditorial tests do not alllow exact separation of hereditery and acquired forms of hearing loss, because of phenotypic similarities. Molecular genetic methods based on mutation detection can provide exact diagnosis in the differential diagnostics of hearing disorders

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SUMMARY:

Molecular genetic examinations have considerable advantages in the diagnosis of diseases. Presymptomatic investigations can prevent the formation of the pathological phenotype. Carrier and prenatal diagnosis can play important roles in cases of severe hereditery diseases, where no treatment is available. Population studies can play a role in the clarification of the genetic background of some diseases, and in developing specific unique therapies for individuals and groups. Identified mutations provide new informations on understanding protein functions. I introduce the importance of the above mentioned applications using four monogenic recessive disorders as examples.

Juvenile haemochromatosis is a severe disease of the iron metabolism with an autosomal recessive inheritance. By direct sequencing of the hepcidin and hemojuvelin genes, that are in the background of the disorder, we identified a new point mutation (p.G66X). This finding unambiguously confirmed the diagnosis based on phenotypic data, provided new informations on protein functions, and gave the opportunity to diagnose the hetereozygous status in case of the family members of the patient.

Haemophilia A and B are X-linked recessive disorders, based on factor VIII, and factor IX deficiency, respectively. Genetic testing of the affected thrombotic factors have great importance in carrier and prenatal diagnostics of family members of haemophilia patients. We identified new disease-causing mutations in 14 cases among 24 patients and 3 new mutations among 27 patients by sequencing the *FVIII* gene and *FIX* gene, respectively. In addition to direct mutation detection, we determined the heterozygous states of the polimorfic markers linked to the two genes in the Hungarian population. These results, and the mutation type patterns agreed with published data. We established a complex algorithm based on direct mutation detection and polimorphic marker testing, which is a reliable and cost effective method in the carrier and prenatal diagnostics of Hungarian families with haemophilia. Carrier status was excluded in 83, and confirmed in 46 cases among potentially HA-carrier women, by investigating 167 haemophilia A families. Mutation was detected or at least one infromative polimorphic marker was identified in 188 HA-carriers, preatatal diagnostics was performed in case of 26 male fetuses. With direct mutation detection carrier status was excluded in 11 cases among potentially carrier women in 27 haemophilia B families, and we were able to perform prenatal diagnostics in 12 obligate and in 13 diagnosed carriers.

One in 2000 newborns is affected by hereditery non-syndromatic deafness. Several genes were proved to be in the background of the disease, out of them the mutations of *GJB2* gene, which codes for connexin 26 cause 50% of the cases. The most common disease-causing mutations differ in populations from different origin and locations. The aim of our study was to identify the allele-frequencies of c.35delG and c.167delT point mutations in randomly selected groups, representative for the Romani and Ashkenazi populations from Hungary, and to compare these data to the ones from the control Caucasian population. Based on our results we can conclude, that the Romani population does not differ from the control Caucasian, however, Ashkenazi differ significantly, allele frequencies of this group are similar to other Ashkenazi groups from different locations. Our methods is appropriate in the differential diagnostics of hearing disorders with providing a fast and reliable manner for testing genetic background of the disease.

The results of my work further confirm the importance of molecular genetic and population testing applications in the study of monogenic recessive disorders.