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Review

Genotype–phenotype correlations in Leber hereditary optic neuropathy

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ABSTRACT

Leber hereditary optic neuropathy (LHON), acute or subacute vision loss due to retinal ganglion cell death which in the long run leads to optic nerve atrophy is one of the most widely studied maternally inherited diseases caused by mutations in mitochondrial DNA. Although three common mutations, 11778G>A, 14484T>C or 3460G>A are responsible for over 90% of cases and affect genes encoding complex I subunits of the respiratory chain, their influence on bioenergetic properties of the cell is marginal and cannot fully explain the pathology of the disease. The following chain of events was proposed, based on biochemical and anatomical properties of retinal ganglion cells whose axons form the optic nerve: mitochondrial DNA mutations increase reactive oxygen species production in these sensitive cells, leading to caspase-independent apoptosis. As LHON is characterized by low penetrance and sex bias (men are affected about 5 times more frequently than women) the participation of the other factors—genetic and environmental—beside mtDNA mutations was studied. Mitochondrial haplogroups and smoking are some of the factors involved in the complex etiology of this disease.

1. Introduction

Mitochondrial diseases are disorders affecting the oxidative phosphorylation system. They may be caused by mutations in nuclear as well as mitochondrial DNA. Mitochondrial DNA is a circular molecule encoding 13 respiratory chain proteins, 22 tRNA and 2 rRNA molecules, and is highly compact with no introns and virtually no noncoding regions except for the regulatory one called the D-loop. mtDNA is present in the cell in numerous copies, up to several thousand in some types of cells, and is maternally inherited [1].

Mitochondrial diseases caused by mutations in mitochondrial DNA are generally considered as neuromuscular or even multisystem disorders. This is true for most of them, the most frequent one, affecting about 1 in 20 000 inhabitants in some regions of Europe [2]. Leber hereditary optic neuropathy (LHON) in its typical form is the isolated atrophy of the optic nerve. It occurs mostly in young adults affecting both eyes simultaneously or sequentially over a period of several weeks or months and is associated with painless, acute or subacute vision loss. The severity differs between patients, even between members of the same family: sometimes light perception can be retained and partial vision recovery, even after several years, occurs in some cases [3].

In over 90% of cases, LHON is caused by one of three mitochondrial DNA (mtDNA) missense mutations in genes encoding subunits of NADH dehydrogenase (ND): 11778G>A (ND4), 14484T>C (ND6) or 3460G>A (ND1) [4]. They are commonly present in the organism in a homoplasmic state (all mtDNA molecules have the mutation), but heteroplasmic (mutated and unmutated mtDNA molecules present) cases are also known. The most severe known mutation is 14459G>A, which shows a wide clinical variability mostly causing a LHON plus phenotype (additional neurological features accompanying typical atrophy of the optic nerve) [5] however patients presenting only with classical LHON are also observed. 14459G>A cannot be easily compared to the three above-mentioned common mutations. 3460G>A mutation is considered to be severe, 11778G>A—intermediate, whereas 14484T>C is rather mild [6]. A minority of cases are attributed to other mtDNA mutations mostly in ND genes. Some of them have been observed in several independent families, but there is a large number of potentially pathogenic mutations still needing confirmation [7–9]. The penetrance is low, up to 50% in men and 10% in women. To make things more complicated, the involvement and role of additional genetic factors (like mitochondrial DNA variants) as the modifiers of disease expression is widely disputed [10].

Although in majority of cases LHON mutations lead to isolated optic nerve atrophy, a small group of LHON plus patients exhibit various neurological symptoms like cerebellar ataxia or psychomotor regression with MRI changes and other abnormalities typical for mitochondrial encephalomyopathies of progressive character [11,12]. On the other hand, in a large population of asymptomatic carriers studies of color discrimination have shown color vision loss in men, especially for green and red, which is also typical for patients with fully developed LHON [13].

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For most patients development of the disease starts with a presymptomatic phase characterized by peripapillary microangiopathy with tortuous vessels, which can last for many years and can even be lifelong. In the acute phase, peripapillary teleangiectatic microangiopathy and swelling of retinal nerve fiber layer around the optic disc along with loss of the papillomacular bundle, starting with more sensitive smaller fibers, is observed. After about 6 months, the vision loss usually stabilizes, the optic nerve atrophy is evident and patients enter the chronic phase of the disease. Optical coherence tomography, a noninvasive imaging technique allowing real-time examination of the retina and optic nerve, revealed that the thickness of the retinal nerve fiber layer (RNLF) correlates with the clinical state of the patient, being slightly thicker in unaffected carriers and in subclinical phase in the temporal quadrant, thicker in the acute phase and getting thinner in the chronic phase. Thinning starts again from the temporal quadrant. Similar thinning of RNLF, predominantly in the temporal quadrant, is observed in autosomal dominant optic atrophy patients (ADOA) with mutations in the *OPA1* gene [14–18].

In most of the cases, vision loss is permanent, although spontaneous recovery is sometimes observed. The frequency of visual recovery depends on the type of mutation, being most frequent for the 14484T>C, and on the age of onset—young patients and children, very rarely affected with LHON, have a higher chance of sight improvement [19].

Besides the limited phenotypic effects of the disease, where only the optic nerve is affected, there is one more striking and very characteristic feature of this particular disorder not observed in other mitochondrial diseases: the higher incidence in men. In the literature male to female ratios vary between about 2:1 and almost 8:1 [20] depending on the type of mutation. The ratios calculated from an extensive LHON study by Hudson et al. [6] are about 4:1 for 11778G>A, 6.4:1 for 14484T>C and 2.3:1 for 3460G>A mutation. The basis of this phenomenon is still unknown and an additional X-linked factor has been postulated in several studies but no gene has been found [21–23].

Although LHON is one of the best studied mitochondrial disorders, the exact mechanism of pathology is still not fully known. Since the mitochondrion is the power factory of the cell, the most obvious explanation was that the mtDNA defect leads to a significant decrease in energy production and failure in optic nerve function. As the experimental data were confusing and the rather small decrease in energy production for some mutations could not explain all the observed phenotypic effects, alternative explanations such as increased oxidative stress, apoptosis and altered axonal transport of the organelles have been proposed [24].

2. Energetic failure?

One of the greatest complications in studying biochemistry and physiology of LHON is the fact that the only affected tissues are retinal ganglion cells (RGC) and the optic nerve, which are not easily accessible for in vitro studies, while the mutations are present in the whole body at a very high, even homoplasmic level, giving no clinical phenotype.

For molecular as well as biochemical studies, numerous cellular models have been used: fibroblasts, platelets, lymphoblasts or skeletal muscle [25], but the most consistent results were obtained with cytoplasmic hybrids (called cybrids) commonly used for mitochondrial mutation research. Cybrids are derived from the fusion of cells deprived of mitochondrial DNA by long culture on ethidium bromide (called Rho0 cells) and cytoplasts—enucleated cells containing the analyzed mitochondria originating from the patient's cells (blood platelets, as structures which naturally do not have nuclei, but having mitochondria, can replace the cytoplasts in this method). The resulting cells have nuclei from Rho0 cells, but mitochondria from the patient and allow for analysis and comparison of the effect of different mitochondrial DNA variants in the same nuclear background [26].

The respiratory capacity and complex I activity were tested in cybrids and the lymphoblast cell lines bearing all three most common LHON mutations, 11778G>A, 14484T>C or 3460G>A. 3460G>A and 11778G>A mutations indeed showed a decrease in respiration rate as well as in complex I activity (especially the former was reduced by about 70% in enzymatic assays in 3460G>A cybrids and even more in lymphoblasts), which is consistent with the fact that it is one of the most deleterious LHON mutations. For 11778G>A respiration rate was only moderately but significantly lowered in both cell types but enzymatic complex I activity was altered only in lymphoblasts. No such effect was observed for the mild mutation 14484T>C, where the respiration rate was lowered by about 10–15% in lymphoblasts only with no change in complex I activity [25]. Although these data reflect the connection between the severity of the mutation and the severity of the OXPHOS defect, they cannot explain the pathomechanism of the disease, especially in case of the 14484T>C mutation [25].

A possible explanation was that mitochondria function differently in neuronal cells. Since it is impossible to study affected neurons, cybrids were again used, but this time 3460G>A and 11778G>A mutations were placed in the context of neural NT2 cells [27]. Again the results were not unequivocal. No reduction in membrane potential was observed in both differentiated and undifferentiated states, but the differentiation process appeared to be altered for LHON mutation bearing cells, since the yield of obtained differentiated cells was decreased.

As these studies gave only little support for the bioenergetic theory of LHON pathology, another possible mechanism was suggested.

3. Reactive oxygen species production?

Mitochondria are the biggest reactive oxygen species (ROS) factory in the cell, and ROS may damage the cell via numerous mechanisms. As dysfunction in the respiratory chain can lead to elevated ROS production, the mechanism of LHON mutation influence on cell physiology became an attractive hypothesis [28].

Indeed, the above-mentioned neuronal cybrids revealed that ROS production was increased about 2.5-fold in cells bearing all three common LHON mutations, but the effect was observed only in differentiated cells, and was absent in undifferentiated ones with the same mutations [27]. As LHON is mostly a defect in complex I genes, it is worth mentioning that inhibition of complex I by rotenone leads to oxygen radical overproduction in human skin fibroblasts, to increased complexity of mitochondrial reticulum and to general outgrowth of mitochondria, which may be a compensatory effect [29,30].

At the same time, higher concentrations of rotenone cause apoptotic death of the cells with all the typical hallmarks like DNA laddering, cytochrome *c* release, or activation of caspase 3 by elevated ROS production, although the extent of the phenomenon was dependent on the cell type [30].

4. Apoptosis?

The next obvious question is whether apoptosis is involved in the atrophy of the optic nerve in LHON. There is no straightforward way to answer it. Even limited post-mortem analysis of the optic nerve would not give clues, because neuron degeneration would have occurred many years earlier [31]. Indirect evidence comes from the fact that there are no signs of inflammation in the optic nerve [32].

Are the cells harboring mtDNA Leber mutations more prone to apoptotic cell death? In the very well characterized apoptotic pathway, mitochondria play a central role in amplifying the apoptotic signal, thus apoptosis of cells containing mitochondria with these mutations would be a very reasonable explanation of LHON pathology. There is increasing support for this hypothesis. Cybrids

with 3460G>A and 11778G>A mutations were found to be more sensitive to apoptotic death after treatment with anti-Fas receptor antibodies [32]. A similar effect was observed in the culture of the cybrids on non-fermentable energy sources like galactose. Cell lines with each of the three typical LHON mutations died within a few days, showing nuclear morphology typical for apoptosis as well as DNA fragmentation and cytochrome *c* release—signs of mitochondrial involvement in the process. However, caspase-3 activation was not observed [33]. More detailed studies revealed that the culture on galactose resulted in a significant reduction in ATP production and apoptotic death via a caspase-independent pathway with the sequential release of cytochrome *c*, AIF and EndoG from mitochondria [34,35].

5. Why the optic, but not any other nerve?

The answer to this fundamental question can lie in the complex structure formed by retinal ganglion cells whose axons constitute the optic nerve. The 5 cm long axons turn at a right angle while leaving the eye and entering the head of the optic nerve, then cross the lamina cribrosa [36]. The paths taken by individual axons may vary, depending on the place in which they start. Another interesting morphological feature is myelination: it is present in the part of the nerve located behind the lamina, while it is absent in the head of the optic nerve. The mitochondrial content is higher in the unmyelinated part, reflecting the higher energetic requirements needed to sustain the action potential. In such a complicated structure, some of its parts may be more prone to different damaging factors [36].

Unlike any other part of the nervous system, the retina is exposed to light, which can be responsible for increased ROS production. As ROS production is already increased in cells with LHON mutations, additional light-induced ROS production may lead to the damage of very sensitive Muller glial cells, which are responsible for maintenance of RGC homeostasis [37].

Another characteristic of the RGC cells is that they are sensitive to glutamate levels and die when the expression of excitatory amino acid transporter EAAT1 responsible for glutamate uptake is low. At the same time LHON mutations are known to influence the function of this transporter in cybrid models and cells with these mutations show decreased glutamate levels [37–39].

Comparison of LHON with another genetic disorder affecting the same type of cells—Autosomal Dominant Optic Atrophy frequently caused by mutations in the *OPA1* gene encoding a protein similar to dynamins and responsible for proper mitochondrial cristae formation—is very interesting. Mutations in *OPA1*, although via different mechanisms, lead to OXPHOS defect and uncoupling of the respiratory chain and cause the release of cytochrome *c* and apoptosis. Similar effects obtained through different mechanisms may be an indication of the susceptibility of optic nerve cells to apoptosis, resulting from different factors [40,41].

6. Modifying factors

Reduced penetrance, a high male to female ratio, and the existence of LHON plus cases strongly suggest the involvement of modifying factors—genetic or environmental.

6.1. Genetic factors

The first factor frequently suggested as a genetic modifier is the mitochondrial haplogroup. A haplogroup is the collection of polymorphisms forming a haplotype reflecting the evolutionary history of the mtDNA molecule. Most of the Caucasian mtDNAs belong to, in order of frequency, haplogroups H, U, T, J, K, V, W, X, I and M [42]. Population studies revealed that a very high frequency of haplogroup J is observed in LHON patients with the 14484T>C mutation. A founder event has explained this effect in at least two populations: French–Canadian and Dutch [43,44], but was excluded in Italian 14484T>C carriers. A slightly higher frequency of haplogroup J has also been observed for patients with the 11778G>A mutation, which may suggest that it has a negative influence on the LHON phenotype [45]. Detailed studies and statistical analysis revealed the association of 11778G>A mutation with J2b and J1c subhaplogroups characterized by additional non-synonymous changes in *Cytb* mitochondrial gene and higher penetrance in the J2 subhaplogroup. For 14484T>C mutation, higher incidence and increased penetrance in subhaplogroup J1 versus J2 were observed, while only 1% of cases belonged to a haplogroup other than J. For 3460G>A, the increased risk was associated with haplogroup K, which bears another non-synonymous variant of *Cytb* [6,25].

At the same time, it was observed that in some populations haplogroup J is more frequent in elderly people [46] and can have a protective effect against Parkinson disease [47]. The above observations have led to a hypothesis that the region specific variants forming haplogroups show different bioenergetic properties helping their owners to adapt to the environment in which they are living, while at the same time making them prone to different disorders, which also demonstrate geographic specificity. Wallace [48] has proposed that haplogroup-specific polymorphisms, including those found in haplogroup J, contribute to more uncoupled mitochondria. If this is indeed true, it could help explain why persons with haplogroup J are more susceptible to LHON, while they are more likely to age well.

At the molecular level, haplogroup J D-loop polymorphisms may influence transcription and replication of mtDNA [49]. At the same time, the bearers of another European haplogroup, haplogroup H, seem to have decreased risk of developing LHON [6]. Altogether the influence of the haplogroup on the phenotype may be quite complex in LHON.

In rare situations two mtDNA LHON mutations occur in the same patient. Only 5 such families have been described: 4 with a combination of 11778G>A and 14484T>C and one with 3460G>A and 11778G>A mutations. Although the presence of two mutations would have been expected to result in a more severe phenotype it only seemed to positively influence penetrance and the LHON plus phenotype was observed only in one family with the 11778G>A and 14484T>C mutations. This lack of aggravation of the phenotype by two mutations in subunits of the same mitochondrial complex may indicate that even though the mutations differ in severity, they affect exactly the same process in mitochondria [50–53]. Even the family bearing the two severe mutations 3460G>A and 11778G>A did not have any additional symptoms other than LHON [53].

Because of the imbalance in male to female LHON cases, an X-linked factor was sought. Until now no such modifier gene has been found, but several susceptibility regions have been described, which may suggest the genetic variability of X-linked factors [21–23,54,55].

6.2. Environmental factors

Since known genetic influences cannot explain low penetrance, a search for environmental factors was initiated. The best studied ones are cigarette smoking and alcohol consumption. For a very long time conflicting sets of data existed; some showing the negative influence of tobacco smoking or alcohol on onset or progress of the disease, and some stating they had no effect [56,57].

Recent extensive analysis of more than 400 affected and unaffected LHON carriers revealed that smoking is associated with a higher risk of developing symptomatic disease, and that heavy smokers were more susceptible to it. The effect of alcohol on disease development was not as strong and did not reach statistical significance [58].

In some patients the first symptoms appear after some kind of trauma: physical injury, psychological stress, infection or exposure to toxic factors. This phenomenon is also observed for other mitochondrial diseases and is another proof for interaction between genes and the environment [58–60].

7. In search of therapy

At present there is no causal treatment of mitochondrial diseases, and in the case of Leber hereditary optic neuropathy there is no symptomatic one. Clinicians generally advise patients not to smoke or drink alcohol. Some of the patients receive anti-inflammatory drugs, with no positive effect, often before the final diagnosis is made.

Attempts to find an active factor improving vision in LHON patients are made on the cell culture level as well as in patients and one of the drugs is now in clinical trial phase. The most widely used component is Coenzyme Q_{10} and its synthetic analogue idebenone as both are effective antioxidants used to reduce reactive oxygen species level contributing to the pathology of the disease. Currently a double blind, randomized placebo controlled clinical phase II trial of idebenone is in progress.

Retrospective studies of idebenone and vitamin (vitamin B2 and vitamin C) treatment suggest that although the number of cases of visual recovery has not changed, the interval between the onset of LHON and the beginning of the recovery process was shortened, which was statistically significant. This treatment may be much more effective in preventing the loss of vision in mutation carriers than in actually curing the disease [9,61,62].

There is a potential field for utilizing some other antioxidants, apoptosis inhibiting factors, or a wide variety of different neuroprotective agents which were found to improve the function of RGC cells in different models.

A combination of all clinical, biochemical, physiological and molecular data clearly show that, while mitochondrial DNA mutations are essential for disease development, they are far from being the only cause. The interaction of different genetic and environmental factors is responsible for the expression of this highly variable and still mysterious disorder, placing it among multifactorial rather than strictly mitochondrial diseases.

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