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Review article

Genetic basis of neural tube defects. II. Genes correlated with folate and methionine metabolism

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Abstract. Effective supplementation with folate, which prevents neural tube defect (NTD) occurrence, and high homocysteine levels in the blood of NTD children's mothers suggest that genes involved in folate and homocysteine metabolism can be involved in NTD aetiology. Genes encoding methylenetetrahydrofolate reductase (*MTHFR*) or methylenetetrahydrofolate dehydrogenase (*MTHFD*) belong to the first group. Genes encoding methionine synthase (*MTR*), its regulator – methionine synthase reductase (*MTRR*) and also cystathionine synthase (*CBS*) can be included in the second group. We present a current list of the folate and homocysteine metabolism genes that are known to be involved in NTD and pay special attention to primary and secondary NTD prevention.

Key words: folate, methionine, NTD.

Introduction

Every year in Poland nearly 1.5% of children are born with congenital malformations, among which the most frequent are defects of the central nervous system and the cardiovascular system. Disturbances in the neural tube closure process lead to neural tube defects (NTD). The incidence of disease in Poland is about 2.05-2.68 per 1000 newborns and has remained unchanged within the last 20 years. Every year about 1000 (800-1200) children are born with a neural tube

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defect and almost half of them have myelomeningocele or encephalocele (CZOCHAŃSKA, LECH 1998, LECH 1998). These malformations either cause disability or death. In Poland, the mortality rate from neural tube defects is about 0.89 per 1000 live-born newborns and is higher than in other European countries, like Great Britain (0.08/1000) or Hungary (0.25/1000).

Multigenic inheritance model

About 90% of all NTDs are isolated malformations, determined multigenetically, which arise independently from other foetal defects. Environmental factors have a significant influence on the development of the disease (BRZEZIŃSKI 1998). The probabilities of NTD occurrence cannot be determined unambiguously. The risk of having a child with NTD is higher in consecutive prequancies in one family, but can be decreased by the change of environmental factors (for example: administration of folic acid and other vitamins in the preconceptual period and during the first three months of gestation). They modulate the effect of proteins that are products of genes believed to be involved in neural tube defect development.

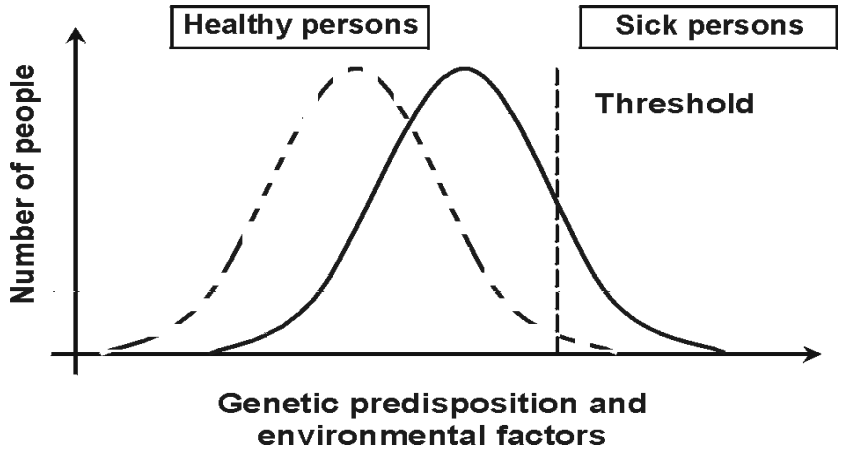

Figure 1. Risk curves for occurence of neural tube defects (dashed line – population curve, black line – families with NTD curve, based on CONNOR, FERGUSON, SMITH 1998)

Falconer and Carter have described the relationship between genetic predisposition, environmental factors and neural tube defects in a model called the threshold model of multigenic inheritance (CONNOR, FERGUSON, SMITH 1998)). According to this model, extreme environmental conditions, which act on a person with specific genetic predisposition, can lead to threshold infringement, after which a specific effect arises, in this case a neural tube defect. If there was no other case of the disease in the family, the risk of its occurrence is the same as in the gen-

eral population and amounts to about 0.1%. If the neural tube defect occurred in the family, so that members of the family have a specific genetic predisposition to a disease, then the susceptibility curve shifts upward which increases the risk of defect development (Figure 1) (CONNOR, FERGUSON, SMITH 1998).

Genes encoding enzymes of folate and methionine metabolism

Neural tube defects can occur as a result of defects in many genes. A high homocysteine level in the blood of women who bear a child with NTD, and effective supplementation with folic acid, which prevents defects, suggest that the genes encoding proteins directly or indirectly connected with folic acid and methionine metabolism, can be involved in the aetiology of neural tube defects.

Folate and methionine metabolism

Folate (pteroiloglutamic acid) is one of the most essential substrates for cell metabolism (Figure 2). Methylated derivates of folate – N10-formyltetrahydrofolate and N5,N10-methylenetetrahydrofolate (5,10-MTHF) – are donors of one-carbon groups for nucleotide synthesis. Another derivate of folic acid – N5-methyltetrahydrofolate (5-MTHF) – produced from 5,10-MTHF by methylenetetrahydrofolate reductase (MTHFR), is a donor of its methyl group for methionine (Met) synthesis from homocysteine (Hcy). This reaction is catalysed by methionine synthase (EC. 2.1.1.13, homocysteine methyltransferase, MS), whose cofactor is cobalamin – vitamin B_{12} (STRYER 1997). The proper activity of methionine synthase depends on the function of methionine synthase reductase (EC. 2.1.1.135, MSR), which participates in changing MS to the active form (LECLERC et al. 1998).

Methionine is metabolised by methionine adenosyltransferase (EC. 2.5.1.6) to S-adenosylmethionine (SAM), which is a donor of methyl groups in many transmethylation reactions. One of the products in these reactions is S-adenosylhomocysteine (SAH), converted by adenosylhomocysteinase (EC. 3.3.1.1) to homocysteine, which can be used again for methionine synthesis.

If the cellular level of SAM is high, the activity of MTHFR is inhibited (the level of 5-MTHF – a substrate in methionine synthesis – is lower) and cystathionine synthase (EC. 4.2.1.22, CBS) is activated. In the reaction catalysed by CBS, homocysteine is metabolised to cystathionine, and then to cysteine (FINKELSTEIN 1998).

Folate deficiency, as well as irregularity inthe function of the homocysteine-metabolising enzyme, produce high levels of Hcy in cells. Homocysteine excess leads to production of betaine, which can be used as a methyl group donor for methionine synthesis. The reaction is catalysed by betaine:homocysteine methyltransferase (EC. 2.1.1.5), an enzyme, which can be found only in kidneys and liver, and its function is correlated with improper activity of methionine synthase. Inhibitors of this enzyme are SAM and SAH – sub-

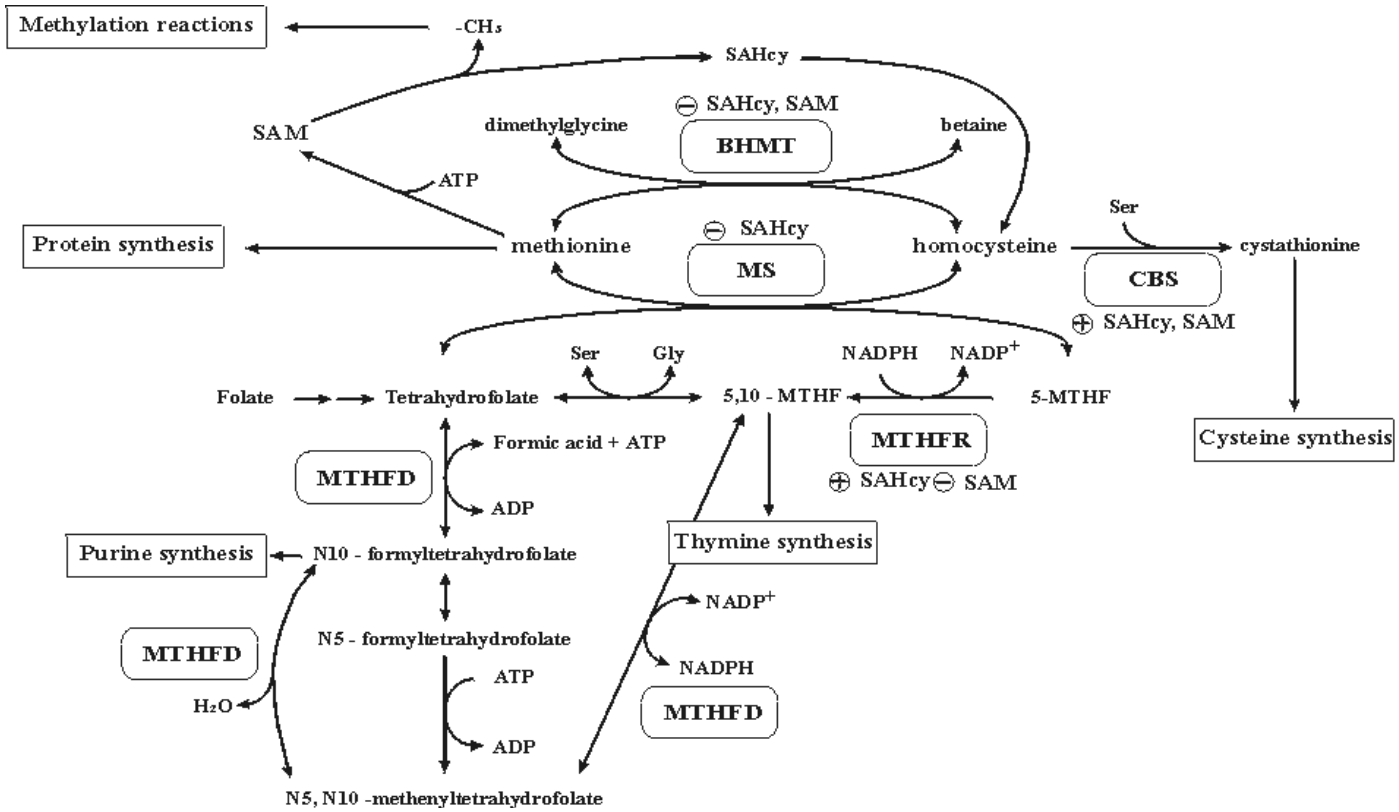

Figure 2. Folate, methionine and homocysteine metabolism

BHMT = betaine-homocysteine methyltransferase, *CBS* = cystathionine synthase, *MS* = methionine synthase, *MTHFD* = methylenetetrahydrofolate dehydrogenase, *MTHFR* = methylenetetrahydrofolate reductase, *5,10-MTHF* = N5,N10-methylene- tetrahydrofolate, *5-MTHF* = N5-methyltetrahydrofolate, *SAM* = S-adenosylohomocysteine, *SAHcy* = S-adenosylohomocysteine, "+" = activator, "–" = inhibitor

stances, which probably regulate the whole homocysteine and methionine metabolism (FINKELSTEIN 1998). In vitro investigations showed that betaine has a toxic effect on cells.

The key enzymes in folate and homocysteine metabolism are: methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MS), cystathionine synthase (CBS) and methionine synthase reductase (MSR).

***MTHFR* – methylenetetrahydrofolate gene**

In 1994, the cDNA encoding human methylenetetrahydrofolate reductase was isolated and sequenced and an amino acid sequence of the enzyme was established. Human MTHFR shares homology with peptides from other species, so it can be used for protein analysis.

Porcine methylenetetrahydrofolate reductase can be found in the cytoplasm in the form of homodimeric structures (2 × 77kDa). The human protein has two isoforms with molecular weight 77 kDa and 70 kDa. The smaller one was discovered only in liver tissues of adult and foetal liver and kidneys (ROZEN 1998). MTHFR has two domains: catalytic one (N terminal end, 40 kDa) binding FAD, NADPH and methylenetetrahydrofolate, and regulatory domain (C terminal end, 37 kDa). Between these domains lies a strong hydrophobic region with amino acid sequence Lys-Arg-Arg-Glu-Glu, which constitutes a cleavage site for trypsin (MATTHEWS et al. 1998). A trypsin digestion of MTHFR does not lead to reductase catalytic activity loss, but makes the protein insensitive to allosteric regulation. The allosteric inhibitor of MTHFR is S-adenosylmethionine (SAM). Also S-adenosylhomocysteine shows a regulatory effect on MTHFR activity. It competes with SAM for protein regulatory sites, but does not have any influence on its activity (FINKELSTEIN 1998).

The gene encoding *MTHFR* has been localized in chromosome 1 (1p36.3) and has 11 exons (Figure 3). Its promoter region has several transcription factor binding sites, but does not have TATA-box sequence. It shares similarity with promoter sequences of other genes coding for example cystathionine synthase, methionine synthase and methionine synthase reductase (HOMBERGER et al. 2000). In exon 1 of *MTHFR* gene there is an alternative splicing site; UTR sequences of this gene are long, which can be correlated with complex gene regulation (GOYETTE et al. 1998).

Investigations on mutations in the *MTHFR* gene focused on the catalytic domain. Twenty-four mutations were identified so far, but most of them have an individual character (private mutations (Figure 3). They are carried by a single person with hyperhomocysteinaemia, which is a risk factor in vascular system diseases and probably in NTD (GOYETTE et al. 1994, GOYETTE et al. 1995, GOYETTE et al. 1996, KLUIJTMANS et al. 1998, SIBANI et al. 2000). Two polymorphisms C677T and A1298C, which slightly change enzymatic activity, have been identified in the *MTHFR* gene.

In case of C677T (A222V) polymorphism, the enzyme becomes thermolabile, causing a loss of its activity with a temperature increase. An equivalent of this mu-

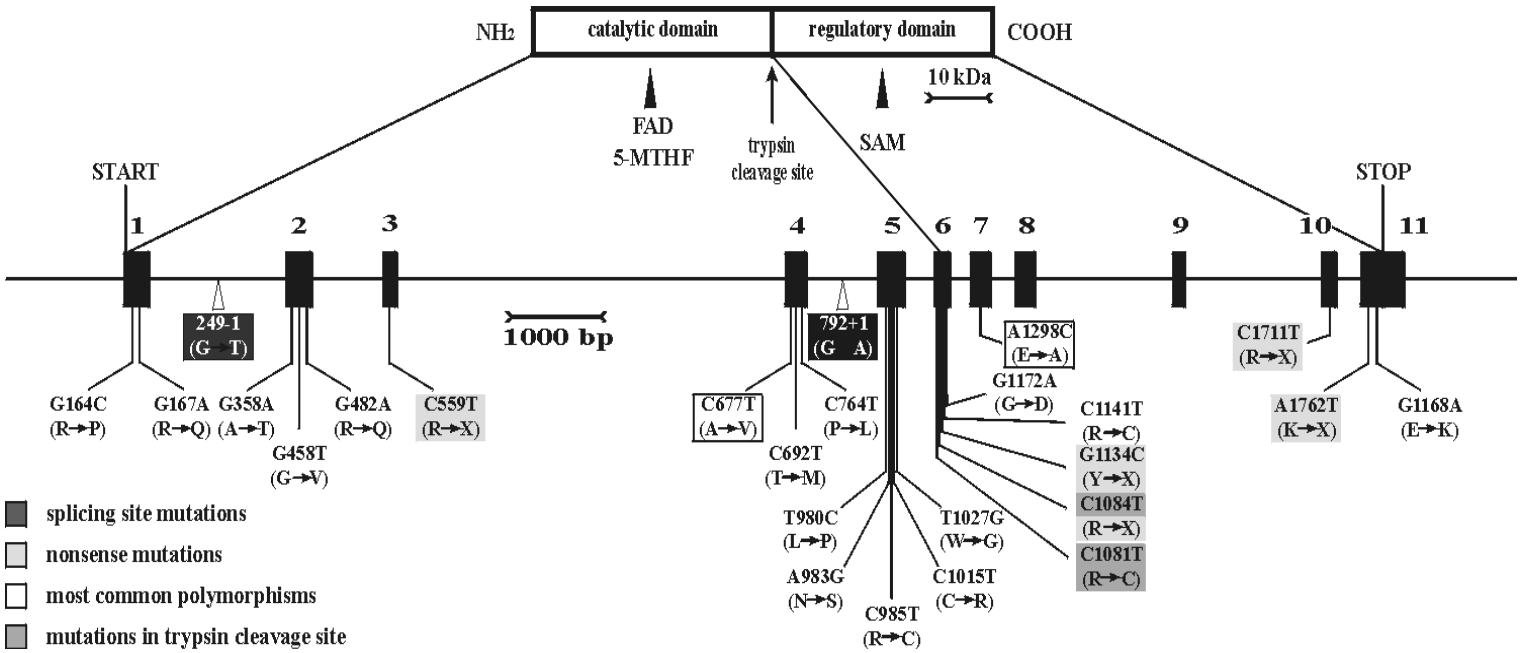

Figure 3. Schematic structure of methylenetetrahydrofolate gene and protein and localization of known mutations

tation in *Escherichia coli* protein is substitution A117V, which is localized near the β8α8 barrel. Probably this mutation changes the secondary structure of the peptide and interactions between monomers. The modified protein loses its cofactor FAD more quickly and has a lower stability. The mutation effect can be suppressed by addition of folate, which causes a higher FAD affinity and an increase in MTHFR stability (HOMBERGER et al. 2000). About 10% of the white population is homozygous for C677TT genotype, and 50% of people have a heterozygous genotype. Frequencies of each allele differ between populations. The frequency of the 677T allele in examined groups from black populations (0.05-0.18) was lower than in, e.g., European populations (0.25-0.44) (BOTTO, YANG 1999).

A1298C polymorphism, localized in the coding regulatory region domain, does not have such effects on protein activity. Results of other authors show that the frequency of 1298CC genotype is about 10% and 1289C allele frequency about 0.36 among distinct populations (BOTTO, YANG 1999).

Decreased enzymatic activity can be also a risk factor for other diseases, like vascular disease, venous thrombosis or oral and cleft palate. As was suggested it does not favour colon cancer progression, because leads to an increase in the concentration of 5,10-methylenetetrahydrofolate which is a substrate for thymine synthesis from uracil. Lower concentration of 5-methyltetrahydrofolate also has an effect on gene regulation (e.g. cancer suppression genes), because it decreases the level of SAM – a substance essential for methylation reactions (ROZEN 1998).

MTR – methionine synthase gene

Increased levels of homocysteine can also be the result of improper methionine synthase function. The substrate of this enzyme is 5-methyltetrahydrofolate, which is produced in reactions catalysed by MTHFR. The methionine synthase cofactor is cobalamin, which participates in methyl group transfer from 5-methyltetrahydrofolate to homocysteine. Products of this reaction are methionine and tetrahydrofolate, which is taken again to folate reaction cycle.

Cobalamin bound to enzyme can have three forms. The first one (cob(I)alamin) is a strong reductor, which can be oxidized (cob(II)alamin), leading to enzyme inactivation. Enzyme activation is dependent on reductive methylation, which produces the active cobalamin form (cob(III)alamin). Methyl group donor in this reaction is SAM, electrons are taken from NADPH and the whole process is catalysed by methionine synthase reductase (LECLERC et al. 1998, MATTHEWS et al. 1998).

The human gene encoding methionine synthase (*MTR*) has been localized in chromosome 1 (1q34) in the telomeric region. The gene is composed of 12 exons and encodes a protein made of 1265 amino acids, so the approximate molecular weight of the protein is 140kDa. Methionine synthase has three domains. The N-terminal domain binds substrates for reaction, the central domain is strongly conserved and binds cobalamin, while the and C-terminal domain is a regulatory domain, interacting with SAM (MATTHEWS et al. 1998, CHEN et al. 1997).

Methionine synthase is conserved among different species. The human form of the enzyme shows 55% of homology with the enzyme from *E. coli* and 66% with the protein from *Caenorhabditis elegans*. It allows for theoretical estimation of mutation effect on the basis of the three-dimensional structure of the bacterial protein (LI et al. 1996).

The most common polymorphism in the *MTR* gene is substitution A2756G, which leads to a change of aspartic acid to glycine (D919G). Aspartic acid is a strong polar amino acid and in this case is localized in the α-helix between two domains: binding cobalamin and the regulatory domain. This region is also responsible for binding proteins that take part in cofactor reduction. The change to non-polar amino acid (glycine, called helix breaker) leads to perturbations in the three-dimensional structure of the protein and also in its function (VAN der PUT et al. 1997). D919G polymorphism probably leads to an improper cofactor oxidation level, which can decrease methionine synthase activity and increase the cellular homocysteine level (CHEN et al. 1997).

MTRR – methionine synthase reductase gene

Methionine synthase reductase is responsible for methionine synthase regulation by reductive methylation. MSR is a member of the electron transferase family and has three characteristic sites, which bind FMN, FAD, NADPH. Isolated mRNA for MSR has about 3.6 kb in length and encodes 698 amino acids. The predicted molecular weight of methionine synthase reductase is about 77 kDa. Probably the enzyme is a cytoplasmic protein. Disturbances in catalytic activity can lead to higher levels of homocysteine, and this can be a risk factor for NTD (LECLERC et al. 1998, WILSON et al. 1999a).

MTRR gene has been localized in chromosome 5 (5p15.2 – p15.3) and is a housekeeping gene, because its expression is low and almost the same in all examined tissues. The gene is made of 15 exons (10% of the whole gene) and together with introns has the size of about 34 kbp. A minisatelite region is located in the 5' end of intron 6, built from 5, 9 or 10 repeats of 29 nucleotides. The *MTRR* gene promoter region does not have any TATA box and its RNA can be alternatively spliced (LECLERC et al. 1999).

The most common polymorphism in methionine synthase reductase gene is A66G substitution, leading to a change of isoleucine to methionine in amino acid 22 (I22M). Although A66G polymorphism does not change the catalytic activity of the protein, the frequency of 66GG genotype is higher in people with NTD and their mothers than in the control group (WILSON et al. 1999b).

CBS – cystathionine synthase gene

Irreversible cystathionine synthesis from homocysteine and serine is catalysed by cystathionine synthase. Disturbances in this process can lead to an increased cellular homocysteine level. Cystathionine is a substrate for cysteine synthesis, which is catalysed by cystathioninase.

Cystathionine synthase is a cytoplasmic protein. The smallest active form is a tetramer, built of four identical monomers with molecular weights of about 63 kDa. More complex structures are made under oxidizing conditions. Each subunit can bind pyridoxal phosphate (PLP, B6 vitamin derivate), SAM molecule (allosteric activator of protein) and heme molecule. The function of the latter has not been explained yet, but probably it takes part in correct protein folding and/or is responsible for enzyme – PLP interactions. The CBS protein has a catalytic core with conserved amino acid sequence (KRAUS 1998, www.uchsc.edu/sm/cbs.html).

The gene encoding cystathionine synthase (*CBS*) has been localized in chromosome 21 (21q22.3) in a region correlated with Down syndrome phenotype. The *CBS* gene is built of 23 exons, but only 1-14 and 16 encode the peptide sequence of 551 amino acids. Alternative RNA splicing could lead to insertion of 14 additional amino acids, which are encoded by exon 15, but a protein like this has not been identified in any examined human tissues. *CBS* has at least two used promoter sequences, but neither of them has a TATA box (KRAUS 1998, www.uchsc.edu/sm/cbs.html).

Ninety-two mutations have been identified so far. Most of them are correlated with homocystinuria and has a missense character. Only 14 mutations were found in more than two alleles, and the others are individual changes. For about 1/3 of all mutations, enzyme activity was analysed. Most of them lead to decreased protein catalytic activity (KRAUS 1999).

Folate receptor genes

Folate receptors are responsible for 5-methyltetrahydrofolate binding and its transport. Inadequate receptor function can lead to lower 5-MTHF transport, and a decrease in intracellular concentration of active folate forms. Folate receptor is a glycoprotein (molecular weight 38-44 kDa) anchored in cellular membrane with a glycophosphatidylinositol domain and takes part in potocytosis[1]. The receptor protein has a site responsible for binding of 5-MTHF, and on its N-terminal the end signal sequence is present, which directs the folate receptor to the membrane (BARBER et al. 1998). Genes encoding folate receptors have been localized in chromosome 11 (11q13), as well as a soluble 5-MTHF receptor and an unexpressed folate receptor pseudogene (DE MARCO et al. 2000).

Genetic variants of folate receptor encoding genes are very rare and have been identified in only a few patients. Probably these receptors are so essential during embryogenesis that their incorrect function leads to foetal death. However it cannot be excluded that mutations of folate receptor genes correlate with neural tube defects, because only the promoter regions and coding sequences were examined (BARBER et al. 1998, HEIL et al. 1999, DE MARCO et al. 2000).

[1]Potocytosis – independent of ATP mechanism by which molecules and macromolecular complexes are sequestered and transported by caveolae (flask-shaped plasma membrane specializations characterized by a filamentous coat).

***MTHFD* – methylenetetrahydrofolate dehydrogenase gene**

MTHFD is another enzyme involved in folate metabolism. This enzyme has three activities: NADP-dependent methylenetetrahydrofolate dehydrogenase, methylenetetrahydrofolate cyclohydrolase (N-terminal end of protein) and ATP-dependent formyltetrahydrofolate synthase (C-terminal end of protein). The gene that encodes MTHFD, has been localized in chromosome 14 (14q24) and has regions coding domains with catalytic activities mentioned above (HOL et al. 1998).

Mutational analysis of *MTHFD* gene in patients with NTD has led to the discovery of G878A substitution in one allele in a patient with familial NTD (*spina bifida*). The mutation was responsible for an arginine to histidine substitution (R293H) in the MTHFD protein. The mutation was also found in the patient's brothers (one had *spina bifida occulta*). In several patients with isolated NTD a substitution G1958A connected with ariginine to glutamine change (R653Q) was observed. The mutation has similar frequencies in control and patients groups, so it is impossible to determine its influence on NTD aetiology (HOL et al. 1998).

Mouse model lines for genes connected with folate and methionine metabolism without NTD

Mutations in genes correlated with folate metabolism, which lead to an increase in homocysteine level in the organism, are not only a risk factor for NTD development. It has also been suggested that they are correlated with vascular system diseases (MATTHEWS et al. 1998, VAN der PUT et al. 1997, DE FRANCHIS et al. 1998, FROSST et al. 1995, HEIJMANS et al. 1999). To check if higher homocysteine levels can be a risk factor for diseases of blood vessels (e.g. coronary vessels) and disturbances in neurulation, mouse lines were established, with one or two deleted alleles of the *Mthfr* or *Cbs* gene. In both cases, live breed was born, but it showed growth delay, increased homocysteine levels and other anomalies. Mice lacking the *Cbs* gene, had a shorter survival period (about 5 weeks) and inadequate liver function, but they did not show neural tube defects. Heterozygous mice are a good model for studying the influence of hyperhomo- cysteinaemia on vascular system function (WATANABE et al. 1995, DAYAL et al. 2001).

Mice heterozygous for the deleted *Mthfr* gene allele develop normally in contrast to individuals without an active form of methylenetetrahydrofolate reductase, which lived shorter and showed movement disturbances. Additionally, in some homozygotic cases, tail deformities were observed, resembling those observed in curly tail mice, which are a model for neural tube defects. Moreover, mice without the *Mthfr* gene had bulging eyes and defective built of the facial part of the skull. In some individuals kyphosis was identified. None of the mice had an evident form of neural tube defects (CHEN et al. 2001).

Murine proteins FBP-1 (folate-binding protein) and FBP-2 are homologues of human folate receptors (FR-α and FR-β). Mice without the gene encoding FBP2

or with only one FBP1 allele have lower folate concentrations, as compared to wild type mice. Mouse foetuses with a knockout *Fbp1* gene die during gestation and show defects in neural tube closure. This suggests that mutations in folate receptor genes can be involved in NTD aetiology (HEIL et al. 1999).

Environmental factors in aetiology of NTD

Beside a genetic basis, environmental factors affect the occurence of NTD. They modulate gene expression, which is confirmed by different frequencies of NTD cases in different geographical regions. NTD frequency is higher in groups with lower economic status and on territories where people are subjected to constant food deficiency. It is known that even a small change in vitamin supplementation can influence gene expression. This is supported by a high efficacy of primary prevention of neural tube defects, based on a special diet before and during the first months of pregnancy. Moreover, drinking alcohol, smoking, taking medicines (e.g. antibiotics or anticonvulsants) and the general health state of the mother have some influence on development of neural tube defects (IQBAL 2000).

Prevention of NTD

Because of the high incidence of NTD in different populations and small efficacy of surgical treatment of this disease, its prevention becomes an issue of utmost importance. Only 1% of all NTD cases do not show neurological disturbances, and about 21% die during the first year of life, even after surgical operation. Also, there is no gene therapy for polygenic diseases, especially that the genes responsible for NTD development have not been identified yet.

Primary prevention

Neural tube defect primary prevention should lead to a decrease in neurulation disturbance frequency. It has been established that nutrition deficiency can be responsible for NTD development and that vitamin administration decreases defect frequency. Folate supplementation prevents not only neural tube defects, but also vascular system defects and colon cancer (LAW 2000). It may be useful to introduce a folate supplement to food in populations with low vitamin consumption.

Secondary prevention

Genetic counselling should encompass families, in which at least one child with NTD was born. Diagnostic examinations play a special role in secondary prevention, which allow detection foetal defect. Diagnostics is based on examination of alpha-foetoprotein (AFP) concentration in the mother's blood serum (if the foetus has NTD, AFP concentration is higher) and ultrasonographic tests. The combination of these two methods allows NTD detection in 90% of cases. Women who gave birth to a child with a neural tube defect should be examined for alpha-foetoprotein concentration and acetylocholinesterase (ACh) presence

in amniotic fluid. Additionally, vaginal USG is conducted, to completely exclude the possibility of a foetal defect (KENNEDY 1998).

In case of neural tube defects – a disease with polygenic inheritance – it is difficult to determine their genetic basis. Currently, the molecular analysis is based on mutation (polymorphism) examination in genes, which could be involved in neurulation disturbances. It seems that in the future, NTD diagnosis will be based on single nucleotide polymorphism analysis.

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