THE IMPACT OF AIR POLLUTION ON GENES IN RELATION TO RESPIRATORY MORBIDITY IN CHILDREN

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Abstract
Introduction: We studied the impact of air pollution on newborns from exposed district Karviná and control district of České Budějovice. We hypothesize that benzo[a]pyrene (B[a]P) can induce mutations in the DNA of these newborns, and we studied this possibility using DNA sequencing in those children at the age of 5 years.
Methods: DNA was isolated from buccal smears from 50 children who were born in the years 2013–2014, from each district. Mutations were followed in exon 11 of gene *ALAS2*, exon 15 of *ZNF341* gene, and exon 1 of gene *ORMDL3* by DNA sequencing.
Results: A pilot study verified our proposed procedure by the analysis of gene *ALAS2*, sequencing exon 11. We did not observe any mutation in any sequence.
Conclusions: It is obvious that our method was correct and primers were properly proposed. We did not observe any differences in the frequency of mutations in the exon 11 of gene *ALAS2* between the groups of children from the districts of Karviná and České Budějovice. The sequence in genes *ZNF341* and *ORMDL3* should be further analyzed.

Keywords: *Air pollution; Benzo[a]pyrene; Buccal smears; DNA sequencing; Gene ALAS2; Gene ORMDL3; Gene ZNF342; Respiratory morbidity*

ORIGINAL ARTICLE

INTRODUCTION

In the Czech Republic, the Moravia-Silesian Region is the region most polluted by PM2.5 (particulate matter < 2.5 μm) and c-PAHs (carcinogenic polycyclic aromatic hydrocarbons), as B[a]P (benzo[a]pyrene) is emitted by heavy industry and local heating systems. Therefore the impact of air pollution on newborns was studied in two districts: the exposed district of Karviná (MSR, Northern Moravia) and the control district of České Budějovice (Southern Bohemia) (Sram et al., 2017).

The concentration of PM2.5 was higher in Karviná than in České Budějovice in the summer of 2013 (mean ± SD: 20.41 ± 6.28 vs. 9.45 ± 3.62 μg/m^3, $P < 0.001$) and in the winter of 2014 (mean ± SD: 53.67 ± 19.76 vs. 27.96 ± 12.34 μg/m^3, $P < 0.001$). Similarly, the concentration of B[a]P was higher in Karviná than in České Budějovice in the summer of 2013 (mean ± SD: 1.16 ± 0.91 vs. 0.16 ± 0.26 ng/m^3, $P < 0.001$) and in the winter of 2014 (5.36 ± 3.64 vs. 1.45 ± 1.19 ng/m^3, $P < 0.001$) (Ambroz et al., 2016).

Higher concentrations of B[a]P in the polluted air in Karviná affected the genome of newborns: increased the level of DNA adducts in cord blood (Šrám et al., 2016), oxidative stress in plasma and urine (Ambroz et al., 2016), concentration of OH-PAHs in urine (Urbancova et al., 2017).

It is postulated that high concentrations of PAHs during prenatal development should affect neurobehavioral functions in the children (Blazkova et al., 2020). We hypothesise that B[a]P can induce mutations in the DNA of these newborns, and we therefore studied this possibility using DNA sequencing in those newborns at an age of 5 years.

For our pilot study we followed mutations in three genes – *ALAS2, ZNF341* and *ORMDL3*. Gene *ALAS2* (DELTA-AMINOLEVULINATE SYNTHASE 2, * 301300), synonyms *ALAS-E, ALASE, ANH1, ASB, SIDBA1, XLDPP, XLEPP, XLSA*, is localized on chromosome X in the region Xp11.21 (ALAS2, ©1966–2020). This gene is long approx. 35 thousands of base pairs and contains 11 exons (Surinya et al., 1998). Enzyme coded by the gene *ALAS2* catalyze heme biosynthesis (Astner et al., 2005). In this gene several deleterious mutations were described that may be responsible for the sideroblastic anemia (Cotter et al., 1992) and protoporphyria (Whatley et al., 2008).

Gene *ZNF341* (ZINC FINGER PROTEIN 341, * 618269), synonyms *HIES3*, is localized in man on chromosome 20, in the region 20q11.22 (ZNF341, ©1966–2020). The gene is longer than 67 thousand base pairs (ZNF341, ©2019). The gene contains 15 exons (Béziat et al., 2018; Frey-Jakobs et al., 2018). It encodes transcription factor, which regulate gene *STAT3* expression and central regulation of immune homeostasis (Béziat et al., 2018; Frey-Jakobs et al., 2018). Many mutations were described, which may cause an absence or formation of damaged protein in the homozygotic form. It disturbs the link of protein to gene *STAT3* promotor; its expression is disturbed or completely missing. It leads to repeated infections and the origins of hyper-IgE syndrome (Hyper-IgE recurrent infection syndrome 3, autosomal recessive; HIES3; 618282) (Béziat et al., 2018; Frey-Jakobs et al., 2018). It is an immunodeficient disease, which is characterized by the manifestation of immunologic and nonimmunologic features such as skin exanthema, eczema, abscess, recurrent bacterial and viral infections of skin and lungs, sometimes together with fungal infections (Freeman and Holland, 2008).

Gene *ORMDL3* (ORM1-LIKE PROTEIN 3, * 610075) is localized in man on chromosome 17, in the region 17q21.1 (Hjelmqvist et al., 2002). It contains 6 exons (ORMDL3, ©2020), 3 codes, and more than 2 thousand base pairs (Hjelmqvist et al., 2002). The group of genes *ORM*, including *ORMDL3*, was identified as a potential risk factor for asthma bronchiale in children. ORM proteins may be the cause of negative regulation of sphingolipids synthesis. Changes in the expression of ORM gene or mutations in their phosphorylated locations cause the dysregulation of sphingolipids metabolism. Incorrect regulation of sphingolipids contribute to the development of asthma bronchiale in children (Breslow et al., 2010). Moffatt et al. (2007) postulated that genetic variants regulating *ORMDL3* gene expression are decesive factors for the predisposition of asthma bronchiale in children.

Our research task was to follow the frequency of mutations in the selected parts of genes in five-year-old children in two districts: Karviná and České Budějovice. We put forward a pilot study to optimize methods and evaluate their suitability for further studies. We may hypothesize that polluted air may contribute to more frequent incidence of mutations (in this case induced and not spontaneous mutations).

MATERIALS AND METHODS

The cohorts were created in the summer of 2013 and the winter of 2014 from newborns born in České Budějovice Hospital, Department of Obstetrics and Gynaecology and Department of Neonatology; and in Karviná Hospital, Department of Obstetrics and Gynaecology and Department of Neonatology. Newborns were selected from the normal deliveries (38th–41st week) of non-smoking mothers who signed a written consent. Cohorts included 99 newborns (summer) and 100 newborns (winter) in České Budějovice, and 71 newborns (summer) and 74 newborns (winter) in Karviná. The study was approved by the Ethics Committee of both hospitals and the Institute of Experimental Medicine CAS in Prague.

Between November 2018 and November 2019, 199 mothers from České Budějovice district and 143 from Karviná district who provided samples from their children in 2013 and 2014, were approached to take part in psycho-

logical testing. Undertaking this psychological test was optional. Out of the total 342 potential subjects, 140 refused to take part in the study, and 31 were impossible to contact. In the present study, data from 99 children from České Budějovice and 70 children from Karviná were collected. The final sample therefore included 169 children. In those children, urine was collected to analyze the content of polycyclic aromatic hydrocarbons (PAHs) and buccal smears.

This study was approved by the Faculty of Health and Social Sciences, University of South Bohemia, České Budějovice.

For our pilot study, 50 random samples of children from each district were selected.

DNA was isolated from buccal smears, which were collected by pediatricians during the preventive examination in 2019. DNA was isolated using commercial kits (NucleoSpin, Macherey-Nagel and the Kit for isolation of DNA, Generi Biotech). The final DNA concentration was in the range of 5–100 ng/μl, and was spectrophotometrically determined, using Nanodrop Colibri.

Primers for the amplification of the segment of gene were proposed using the program “Primer-BLAST“, which is publicly accessible at NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast). They were synthetized according to our proposal by the Generi Biotech company. Table 1 represents the sequences of proposed primers for three analyzed regions.

Table 1 – Sequences of proposed primers

Gene	Primer forward sequence (5 ´→ 3´)	Primer reverse sequence (3 ´→ 5´)
ALAS2	TCCCCCTACCACCTTCAGAG	GCCACTTGTCTGAGGGAGTC
ZNF341	TGATGCTCCTGTTTGGGTCC	CACCCGGACACGTTATGACA
ORMDL3	GTTTGCTGTTGTGAGGGCTG	CAGGAAACCACCAGAGACCC

For the analysis, exon 11 of gene *ALAS2* was selected, which contains 289 base pairs, the part of exon 15 of gene *ZNF341* with 728 base pairs, and the part of exon 1 of gene *ORMDL3*, containing 497 base pairs. In Fig. 1 the brown colour represents exon sequences, the red colour primers (which mark boundaries of amplified region), and the black are adjacent intron regions.

Amplification of selected regions was done by the polymerase chain reaction (PCR). PCR was done by the commercial PPP Master Mix including thermostabile polymerase (Top-Bio). PCR reaction was carried out in the total volume of 15 μl. The reactive mixture contained 7.5 μl of PPP Master Mix, 1.5 μl of the forward primer + 1.5 μl of reverse primer at concentration 10 pmol/μl, 3 μl PCR water and 2 μl DNA. The content of water and DNA was modified according to its concentration. PCR reaction proceeded in thermocyclers: early denaturation 10 min. at 95 °C; 35 cycles of denaturation 30 s. at 95 °C, anenealing 30 s. at 60 °C, extension 1 min. at 72 °C; final extension 10 min. at 72 °C. The amplification products were checked using gel electrophoresis at 2% agarose gel, visualized by ethidium bromide under UV light, and recorded by photo documentation. Fig. 2 represents the final electrophoresis of the amplified segment of gene *ZNF341*. PCR products (5 μl) were enzymatically purified (Enzymatic PCR cleanup) using exonuclease (Exo I, 0.5 μl) and Shrim alcalic phosphatase (rSAP, 1 μl) for 15 minutes at 37 °C. Later the temperature was increased to 80 °C to inactivate enzymes.

Sequencing reactions were prepared separately for forward and reverse primers: 5 μl of primer at concentration 10 pmol/μl were mixed with 1.5 μl PCR product (20–80 ng/μl) and 3.5 μl of PCR water. In the case of very strong or as opposite very week band at electrophoresis, the quantity of PCR product was decreased or increased. Tubes were marked by code and send to GenSeq, s.r.o company for sequencing.

Gene *ALAS2*

Atttggaagatctagtctaacccattttttccctcccc**tccccctaccaccttcagag**aagctgctgctggcttggactgcggtggggctgcccctccaggatgtgtctgtggctgcctgcaatttctgtcgccg

tcctgtacactttgagctcatgagtgagtgggaacgttcctacttcgggaacatggggccccagtatgtcaccacctatgcctgagaagccagctgcctaggattcacaccccacctgcgcttcacttgggtc

caggcctactcctgtcttctgctttgttgtgtgcctctagctgaattgagcctaaaaataaagcacaaaccacagcatgtgaagccttttattggacagggaacagacaagtgcattct**gactccctcagaca**

agtggcagatctatgaggtaaccataggtcacttgttggtcaccatt

Gene *ZNF341*

actttcccttgccctccaggcatgaagctccacaaatgcgccctgtgcagcaagtccttcagccgccgtgcccacctcgccgagcatcagcgcgcccacacgggcaactacaagttccgctgtgctggctgc

gccaagggcttttcccgccacaaatacctcaaagatcaccgctgtcgtctcggcccccaaaaggacaaggacctgcaaacccggcggccccccagaggagggcagccccccgcagttgcggcagtggt

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gcccgtctacatccaggcctccgagtgacggacctgaggtgtctgtttcctgggcaggc**ctgatgctcctgtttgggtcc**agggcccctgggggcagaccggtgatccttaccagtggaagcgagccatcga

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tacgatactactacctcatagggttgtcctggggatccagtatgatgaagtgcgccaggggcttggcatggtgcccggcacgcagaaagtgctcaataaatgtttt**tgtcataacgtg**tccgggtggcctggg

Gene *ORMDL3*

gttttgtgctcctcttccagaagacaggag**gtttgctgttgtgagggctg**ctctttcctgggggagggaatgggaagggctcacctggtggctgccctggtggtgctcagccttggcttgagtgtctggcatac

tggctgggcccggatatttgtatgtggagccaagagggtggggcgggaaggagggggaaagagctaccagcaaaaaagtggttaacacaacagagttgctgcaggctctggtttgaggcaggttatga

aaccacatcagggcccagcactaagcagccacctgccagctatggcagcagaagttttcggctcaactcctgtagacctagtcgacctctgccagcctcttctctctgtttctccccatctagggtaggggcc

agttggttgttttactaatggaagtgatatgtatccatagtcacacgaaagtctcaagagtgtttcctcattgccagtctttttgttagtttattgatctcttctttttcag**gggtctctggtggtttcctg**ctctcag

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tctgggattccccaaggcttttggcactcctggacaaatactaggaagggttagtggactttctataccagcacccctgtaggtgcgggttacagagaggtgatggggtggcagtttcaggtcagacttggg

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cccaggctggcgcacgggtgtttgcttgttgggatggcgtgttgtgggtcacattttctctgaggtctctaagcttggagctgtctgagaagaggaaacttgctcctgttatcctggaagtaaataactggccc

ctgaggactcagtctcctgtgcagggccctcagggatctggggacagccaaactctggataaggggcagatcattccacccccttccttctctacttccctctgctcactgtgggttgcggccacctcctggttc

Fig. 1 – Sequences of analyzed segments

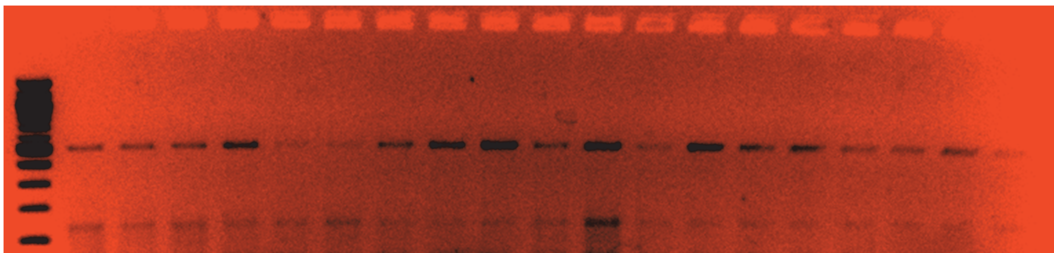

Fig. 2 – Example of PCR electrophoresis for gene *ZNF341*

RESULTS

Final sequencing was released in the format FASTA, PDF and ABI at the address www.eurofinsgenomics.eu. From each sample, two sequences (forward and reverse) from three gene segments were obtained (600 sequences in total). They were evaluated using the program BioEdit 7.2.

Our results were then compared with the reference sequences obtained from the Program BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) at the website NCBI (National Center for Biotechnology Information) – Fig. 3:

1. Homo sapiens 5'-aminolevulinate synthase 2 (ALAS2), RefSeqGene (LRG_1163) on chromosome X; Sequence ID: NG_008983.1
2. Homo sapiens zinc finger protein 341 (ZNF341), RefSeqGene on chromosome 20; Sequence ID: NG_053072.1
3. Homo sapiens ORMDL sphingolipid biosynthesis regulator 3 (ORMDL3), transcript variant 1, mRNA; Sequence ID: NM_001320801.1

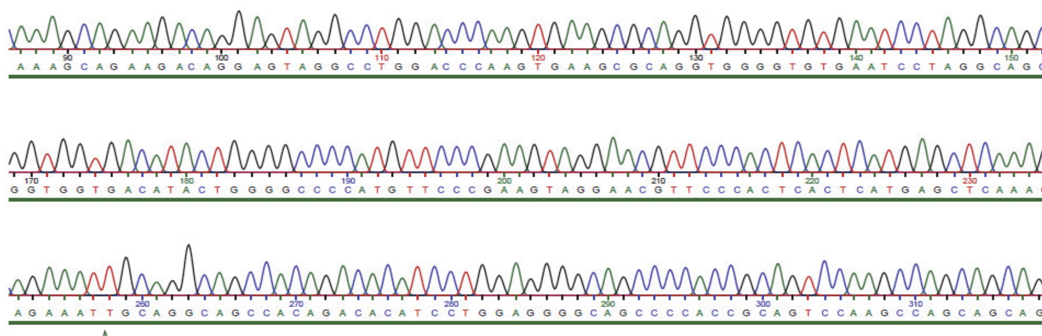

Fig. 3 – Example of obtained sequencing

The pilot study verified our proposed procedure by the analysis of gene *ALAS2*. We analyzed the sequence of exon 11 and did not observe any mutation in any sample.

DISCUSSION

Our results indicate that the collection of buccal smears acquired from children is a convenient non-invasive method to obtain DNA. We verified the convenience of proposed primers as well as used procedure.

The frequency of mutations differ among species, as well as according to the environment in which an organism occurs. Chemical compounds in our environment represent a danger for living subjects, particularly due to their toxicity and mutagenic effect. Benzo[a]pyrene (B[a]P) is the proved human carcinogen. A concentration of B[a]P in polluted air higher than 1 ng/m^3 induces genetic damage (WHO, 2010). Mutations are the results of changes in the sequence of base pairs in DNA or induction of DNA adducts, which prevent the correct DNA replication and/or transcription of DNA to RNA. We may hypothesize that the origin of diseases, including cancer, may also be caused by the accumulation of mutations in DNA from chemical pollution in our environment.

We hypothesized that increased B[a]P exposure in the district of Karviná could increase the frequency of mutations in genes, related to respiratory diseases such as, for example, asthma bronchiale, as we previously observed in this region (Sram et al., 2013). But we did not see any differences between children born in the exposed and control districts. This outcome may be affected by the number of examined children. Our sample corresponded to our original idea to complete a pilot study, where we checked the convenience of the proposed procedure: isolation of

DNA from buccal smears, selection of genes related to asthma bronchiale, proposed primers and sequence analysis.

Somatic mutations in skin cells may be induced by environmental factors (Saini et al., 2016). The primary material for DNA isolation were cells obtained by buccal smears. Therefore, we cannot claim that our negative results in the cells from buccal smears correlate with the incidence of somatic mutations in other parts of body.

CONCLUSIONS

We may conclude – (and it is quite obvious) – that our method was correct and the primers were properly proposed.

We did not observe any differences in the frequency of mutations in the exon 11 of gene *ALAS2* between the groups of children from the districts of Karviná and České Budějovice.

The sequence in genes *ZNF341* and *ORMDL3* should be further analyzed.

Conflict of interests

The authors have no conflict of interests to declare.

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