DEVELOPMENT AND APPLICATION OF BIOMARKER METHODS IN MOLECULAR ENVIRONMENTAL EPIDEMIOLOGY FOR THE DETECTION OF EXPOSURE TO POLYCYCLIC AROMATIC HYDROCARBONS

PhD theses

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Introduction

Influence of environmental xenobiotic compounds to the human body can be followed up at several points along the pathway from the exposure to the adverse health effects. From biological samples, the level of exposure to a given compound can be investigated by using biomarkers of exposure, and the early and late effects are measureable by the biomarkers of effects. The biologically effective dose of a potential carcinogen in a given tissue can be characterised by the amount of DNA adducts, that are formed with covalent binding between the genotoxic agent and the DNA.

The DNA adduct biomarker reflects the individual exposure, and allows for more accurate exposure assessment than the concentration of the potential cancer-causing compound in the environmental samples, thereby improves the efficiency and the accuracy of the epidemiology studies. A number of DNA adducts are pre-carcinogenic DNA alterations, therefore their measurement from human DNA is of great importance in the research of the mechanism of action of potential cancer-causing environmental agents. DNA adducts are applied for the detection of various types of exposure including environmental, dietary, medical, and occupational exposures.

The methods for the determination of DNA adducts vary in their sensitivity and in the required amount of DNA substantially. The most widely used methods for the measurement of polycyclic aromatic hydrocarbon (PAH)-DNA adducts, are ^{32}P-postlabelling and immunoassays that provide semi-quantitative determination. The ^{32}P-postlabelling technique has wide substrate specificity and high sensitivity, therefore it is especially advantageous in molecular epidemiological studies for the detection of complex environmental exposures, particularly PAH exposure. In the ^{32}P-postlabelling method, the DNA adducts are labelled with a radioactive isotope – phosphorus-32 of high specific radioactivity – and the radioactive decay is detected. The detection limit of ^{32}P-postlabelling is about 1 adduct in 10^{10} nucleotides from 10 μg DNA. For the determination of PAH-DNA adducts, benzo[a]pyrene-7,8-diol-9,10-epoxide (BPDE)-DNA immunochemical methods are used, in which an

antiserum/antibody elicited against DNA modified *in vitro* with BPDE is applied. The antibody recognises not only the BPDE-DNA adducts, but due to cross-reactivity of the antibody, also chemically-related other PAH-DNA structures. In the ELISA, DELFIA and CIA competitive immunoassays the measured signal is bound to the DNA adducts. The detection limit of the competitive immunoassays is 1 to 10 adducts in 10^8 nucleotides in 10 to 35 μg of DNA.

Both types of these high-sensitivity methods are suitable for the investigation of human samples. However, their technical complexity, limited automation opportunities, and demanding manpower and skill are limiting factors for the analysis of several hundred or several thousand human biological samples in large-scale molecular epidemiological projects. Therefore there is a great demand of development of high-sensitivity and high sample-capacity economical biomarker methods.

Aims

The main objective of my PhD work was the further development and validation of the ^{32}P-postlabelling method and an immunochemical assay for the detection of environmental PAH exposure by the measurement of DNA adducts.

Further development of the ^{32}P-postlabelling method in order to:

- increase the through-put of the ^{32}P-postlabelling method substantially without increasing the total radioisotope requirement, whilst maintaining radio-safety of the laboratory personnel;
- elaborate a method that is generally applicable for the DNA adduct analysis of various types of DNA samples.

Validation of the newly developed high through-put direct sandwich chemiluminescence immunoassay (BPDE-DNS SCIA) for the determination of PAH-DNA adducts by:

- comparisons of DNA adduct quantification by the immunoassay and the ^{32}P-postlabelling method from different types of DNA samples;
- the investigation of the correlation of the DNA adduct levels between the two methods.

Investigation of the impact of the preparation of the biological samples (storage, DNA isolation) to the quantification of DNA adducts by the ^{32}P-postlabelling method and the new SCIA.

Materials and Methods

DNA samples:

i) BPDE–DNA adduct standard;

ii) Cell culture – MCF-7 cell line treated in aliquots of 2 x 10^7 cells with 1 μM benzo[a]pyrene (B[a]P) at 37 °C for 24 hours;

iii) Treated mice: CD2F1 mice treated with intra-peritoneal injections of B[a]P (100 and 200 mg/kg), benzo[b]fluoranthene (B[b]F) (200 and 400 mg/kg), and dibenzo[a,h]anthracene (DB[a,h]A) (2,5; 5 and 10 mg/ kg), respectively. The DNA samples isolated with Qiagen Midi Kit were provided by Dr. Soterios Kyrtopoulos (NHRF, Athen, Greece);

iv) DNA samples from human peripheral blood lymphocytes and non-tumorous peripheral lung tissue samples;

v) Buffy coat fractions from healthy human maternal peripheral blood and newborn cord blood.

DNA isolation:

DNA was isolated from aliquots of the treated MCF-7 cells and from human biological samples with the traditional phenol – chloroform - iso-amyl alcohol extraction procedure, a modified Qiagen Midi kit procedure and the salting out procedure.

DNA adduct determination by the regular ^{32}P-postlabelling method:

DNA (4 μg) was digested with micrococcal nuclease and spleen phosphodiesterase to mononucleotides, followed by adduct enrichment with nuclease P1, then the radio-labelling of the adducts occurred with 50 μCi [γ-^{32}P]ATP and T4 polynucleotide kinase. Separation of DNA adducts was performed by multi-directional thin-layer chromatography. Radioactivity patterns were detected and

quantified by electronic autoradiograph. Background radioactivity in the blank area, corrected for the size of the adduct areas was subtracted from the radioactivity of the adduct areas.

The modified ^{32}P-postlabelling method:

DNA digestion (4 μg) and the adduct enrichment were the same as in the regular method. An evaporation-to-dryness step of the DNA digest was introduced before the ^{32}P-radiolabelling step. The amount of the radioisotope was decreased from the regular 50 μCi per 4 μg DNA in a range down to 10 μCi per 4 μg DNA sample. The final reaction volume in the radio-labelling step was set to 5 μl. The chromatographic separation, the detection of the radioactivity patterns and correction for the background radioactivity was the same as in the regular method.

DNA adduct determination by the newly developed BPDE-DNA SCIA method:

The DNA sample was denatured to single stranded DNA and fragmented to specific-size fragments (2000 bp/fragment) by restriction enzyme. The adducted DNA fragments were selectively bound to a solid surface (microtiter plate) by a specific rabbit antiserum elicited against BPDE-DNA standard. The chemiluminescent signal to be measured was coupled to the non-adducted mononucleotides by a monoclonal DNA-antibody. BPDE-DNA standard curve was used for the calculation of the levels of PAH-DNA adducts.

Statistical analyses:

DNA samples were analysed in two replicates by the ^{32}P-postlabelling method and in three replicates by BPDE-DNA SCIA. The statistical analyses were performed with GraphPad Prism 4.0 software, using Mann-Whitney, Wilcoxon and paired t tests. The correlation analyses were performed with Spearman correlation test. Two-tailed p values were given. $P < 0.05$ was considered statistically significant.

Summary of results

My research aimed at the further development of the ^{32}P-postlabelling method by which, whilst maintaining the adduct-labelling efficiency, the amount of the radioisotope per sample can be reduced, and thereby the sample processing capacity of the laboratory is increased.

The regular and the modified ^{32}P-postlabelling methods were used for comparative determinations of the levels of DNA adducts from various types of DNA samples, i.e. BPDE-DNA adduct standard, DNA samples from MCF-7 cell line treated with B[a]P, DNA samples from human non-tumorous peripheral lung tissue, human peripheral blood lymphocytes and human buffy coat samples. In the modified ^{32}P-postlabelling method, the final reaction volume of the radio-labelling was reduced with an evaporation-to-dryness step to one-third of the volume that was used in the regular method. This facilitated the reduction of the amount of [γ-^{32}P]ATP substrate from 50 μCi per sample – depending on the DNS isolation method – by 50% for the Qiagen-isolated samples (i.e., 25 μCi per sample) and by 80% for the DNA samples isolated with the classic phenol extraction procedure (i.e.,10 μCi) for both experimental and human samples.

The newly-developed BPDE-DNA direct sandwich chemiluminescence immunoassay (BPDE-DNA SCIA) for the determination of PAH-DNA adducts has been published in 2012. Whereas in the earlier competitive immunoassays the signal to be measured is coupled to the DNA adducts, in the SCIA the chemiluminescent signal is coupled to the non-adducted nucleotides. In SCIA, the strong end-point signal derives from the many orders of magnitude difference between the number of the non-adducted and the adducted mononucleotides. The limit of detection of the method is ~ 1.5 adducts/10^9 nucleotides from 5 μg DNA sample. For the validation of BPDE-DNA SCIA, I performed comparative measurements between the immunoassay and the ^{32}P-postlabelling method. The DNA samples were obtained from MCF 7 cells treated with B[a]P, from the liver of mice, which were treated *in vivo* with several doses of B[a]P, B[b]F, and DB[a,h]A, respectively, and from human maternal peripheral blood and newborn cord blood samples. For the B[a]P-DNA adduct levels measured by SCIA and ^{32}P-postlabelling from the MCF 7 cells,

the ratio between the adduct values was about 0.5. For the animal samples, the adduct levels were several times lower by the immunoassay than by the ^{32}P-postlabelling method (the ratios were ≈1:5 for B[a]P, ≈ 1:30 for B[b]F and ≈ 1:5 for DB[a,h]A). All the same, there was a very strong, highly significant positive correlation between the DNA adduct measurements of the dose-response curves by SCIA and ^{32}P-postlabelling for each PAH compound (r = 0.87-0.99). For the human samples, the ratio between the SCIA and ^{32}P-postlabelling values was approximately 1:10, but there was not correlation between the data-pairs measured by the two methods. For the human samples, the lack of correlation between the two methods may be explained by the different efficiency of detection of different structural types of DNA adducts that are derived from complex human environmental exposure.

New scientific achievements and their application

- With the modification of the ^{32}P-postlabelling method, I increased the through-put of the process – the number of DNA samples that can be worked up in one session – 2 to 5-fold – depending on the method that had been used for the isolation of the DNA. The modified ^{32}P-postlabelling method is generally applicable for experimental and human samples, provides unchanged optimal adduct-labelling efficiency and radio-safety of the personnel.

- Based on the above advantages, I particularly recommend the modified method for use in large-scale molecular epidemiological studies for the detection of environmental PAH exposure.

- I introduced the modified ^{32}P-postlabelling method into the international molecular epidemiological research, and I employed it in NewGeneris EU FP6 Integrated Project No. 016320 (Consortium leader: Prof. Dr. J. Kleinjans, Maastricht University, the Netherlands), for the detection of environmental and dietary PAH exposures in European mother-newborn child cohorts for about 600 samples.

- In the validation of the newly developed direct sandwich chemiluminescence immunoassay, BPDE-DNA SCIA, I demonstrated that this method, similarly to the ^{32}P-poslabelling method, recognises the differences of PAH exposure among the exposure groups. From this qualitative point of view, the SCIA is equivalent to the ^{32}P-postlabelling method, and is suitable for experimental and molecular environmental epidemiological studies. For the individual samples, the lack of correlation between the two methods confirms our knowledge of the partial difference/overlapping of the substrate spectra of the two methods.

- When writing this doctoral dissertation, the BPDE-DNA SCIA has already been employed for about 2,000 maternal and newborn blood samples from European cohorts in the NewGeneris EU FP6 Integrated Project No. 016320, for the detection of maternal and fetal PAH exposure. The results of the statistical analyses and the consortium publications are currently being prepared.

- I showed that in the sample preparation process, the isolation methods and the storage conditions of the DNA samples exert a critical impact on the quantitative determination of DNA adducts. Therefore, a special attention should be placed to this factor when archived DNA samples are used in molecular epidemiological studies, and also when DNA biorepository banks are established for future studies.

Publications related to the PhD work in scientific journals and books:

Georgiadis P, Kovács K, Kaila S, Makedonopoulou P, Anna L, Poirier MC, Knudsen LE, Schoket S, Kyrtopoulos S. (2012) Development and validation of a direct sandwich chemiluminescence immunoassay (SCIA) for measuring DNA adducts of benzo[a]pyrene and other polycyclic aromatic hydrocarbons. Mutagenesis, 2012 May 18. [Epub ahead of print] ***Impact factor: 3.98***

Kovács K, Anna L, Rudnai P, Schoket B. (2011) Recovery of bulky DNA adducts by the regular and a modified ^{32}P-postlabelling assay; influence of the DNA-isolation method. Mutat. Res., 721:95-100. ***Impact factor: 2.94***

Gallo V, Khan A, Gonzales C, Phillips DH, Schoket B, Győrffy E., Anna L, Kovács K, Möller P, Loft S, Kyrtopoulos S, Matullo G, and Vineis P. (2008) Validation of biomarkers for the study of environmental carcinogens: a review. Biomarkers, 13: 505-534. ***Impact factor: 1.73***

Győrffy E, Anna L, Kovács K, Rudnai P, Schoket B. (2008) Correlation between biomarkers of human exposure to genotoxins with focus on carcinogen DNA adducts. Mutagenesis, 23:1-18. ***Impact factor: 3.16***

Kovács K, Győrffy E, Anna L, Schoket B. (2006) Környezeti policiklusos aromás szénhidrogén expozíció biomonitorozása vizelet 1-hidroxipirén tartalmának meghatározásával – gyermekekre és felnőttekre vonatkozó szakirodalmi adatok összehasonlítása. Egészségtudomány, 50: 188-193.

Kovács K, Győrffy E, Anna L, Schoket B.: 1-Hydroxypyrene. In: Epidemiological concepts of validation of biomarkers for the identification/quantification of environmental carcinogenic exposures. Eds. Vineis P, Gallo V. The Nofer Institute of Occupational Medicine, Lodz, Poland, ECNIS 2007.

Győrffy E, Anna L, Rudnai P, Kovács K, Schoket B.: Correlations among biomarkers. In: Biomarkers of carcinogen exposure and early effects, Eds. Farmer PB, Emeny JM, The Nofer Institute of Occupational Medicine, Lodz, Poland, ECNIS, 2006.

Publications beyond the subjects of the dissertation:

Anna L, Kovács K, Győrffy E, Schoket B, Nair J. (2011) Smoking-related O^4-ethylthymidine formation in human lung tissue and comparisons with bulky DNA adducts. Mutagenesis, 26: 523-527. ***Impact factor: 3.98***

Anna L, Holmila R, Kovács K, Győrffy E, Győri Z, Segesdi J, Minárovits J, Soltész I, Kostič Sz, Csekeő A, Husgafvel-Pursiainen K, Schoket B. (2010) *TP53* tumorszupresszor génmutáció vizsgálatok magyar tüdőrákos betegcsoportban. Egészségtudomány, 54: 56-69.

Anna L, Holmila R, Kovács K, Győrffy E, Győri Z, Segesdi J, Minárovits J, Soltész I, Kostič Sz, Csekeő A, Husgafvel-Pursiainen K, Schoket B. (2009) Relationship between *TP53* tumour suppressor gene mutations and smoking-related bulky DNA

adducts in a lung cancer study population from Hungary. Mutagenesis, 24: 475-480. ***Impact factor: 3.54***

Abstracts in scientific journals*:*

Kovács K, Anna L, Győrffy E, Schoket B: The impact of pre-analytical processing of human tissue samples on the measurement of bulky DNA adducts by ^{32}P-postlabelling. Mutagenesis vol. 26, no. 5, pp. 689–722, 2011. ***Impact factor: 3.98***

Anna L, Kovács K, Lukács V, Győrffy E, Rudnai P, Schoket B: Assessment of exposure to polycyclic aromatic hydrocarbons during pregnancy with bulky DNA adduct biomarker in European mother - child cohorts. Mutagenesis vol. 26, no. 5, pp. 689–722, 2011. ***Impact factor: 3.98***

Rudnai P, Varró MJ, Rudnai T, Náray M, Schoket B, Anna L, Györffy E, Kovács K, Ürömi J, Herczegh T, Bodnár J: Associations between the children's blood lead level and their health status. Epidemiology 20(6):S260, 2009. ***Impact factor: 5.5***

Anna L, Holmila R, Kovács K, Győrffy E, Győri Z, Segesdi J, Minárovits J, Soltész I, Kostič Sz, Csekeő A, Husgafvel-Pursiainen K, Schoket B: A *TP53* génmutáció és az aromás DNS-addukt szint közötti összefüggés tüdőrákos dohányzóknál. Magyar Onkológia 53: 6, 2009.

Kovács K, Győrffy E, Anna L, Schoket B: Urinary 1-hydroxypyrene as a biomarker of polycyclic aromatic hydrocarbon exposure in the general population and correlation between 1-hydroxypyrene and white blood cell DNA adducts - Results of our literature survey, European Journal of Molecular and Genetic Toxicology http://www.swan.ac.uk/cget/ejgt1.htm, 2006.

24 *international and national conference participations as first author with poster and/or oral presentations.*
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