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Ph.D. Thesis

Cytogenetic analysis using fluorescence in situ hybridization (FISH) to evaluate occupational exposure to carcinogens

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R.J.Sram, **O.Beskid**, B.Binkova, I.Chvatalova, Z.Lnenickova, A.Milcova, I.Solansky, H.Bavorova, D.Ocadlikova, P.B.Farmer: Chromosomal aberrations in environmentally exposed population in relation to metabolic and DNA repair genes polymorphisms. *Mutation Research 2006* – submitted.

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R.J.Sram, P.Rossner, J.Rubesš, **O.Beskid**, Z.Dusek, I.Chvatalova, J.Schmuczerova, A.Milcova, I.Solansky, H.Bavorova, D.Ocadlikova, O.Kopecna, P.Musilova: Possible genetic damage in the Czech nuclear power plant workers. *Mutation Research* 593 (2006) 50-63.

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O.Beskid, Z.Dusek, I.Solansky, R.J.Sram: The effects of exposure to different clastogens on the pattern of chromosomal aberrations detected by FISH whole chromosome painting in occupationally exposed individuals. *Mutation Research* 594 (2006) 20-29.

1. Introduction

1.1. Objective of molecular epidemiology and the use of biomarkers

The epidemiology studies the determinants of human diseases - identifies their causes, determines outcomes and develops prevention strategies. A major objective of **molecular epidemiology** is to provide reliable and specific information about the etiology and mechanism of disease process in order to prevent genesis of the disease. This field of molecular epidemiology is based on the use of biomarkers. **Biomarkers** are measurable biological parameters reflecting, in some way, an individual's risk of disease, because they represent events that appear on direct pathways leading from the initiation to the occurrence of a disease. It means that they may indicate either the exposure to a causative (or protective) agent, or the presence of disease at early stage of development, and allow an assessment of individual susceptibility. The use a biomarkers instead of classical endpoints of epidemiology, such as disease incidence or mortality, represents thus very promising approach which could significantly contribute to the improvement of public health.

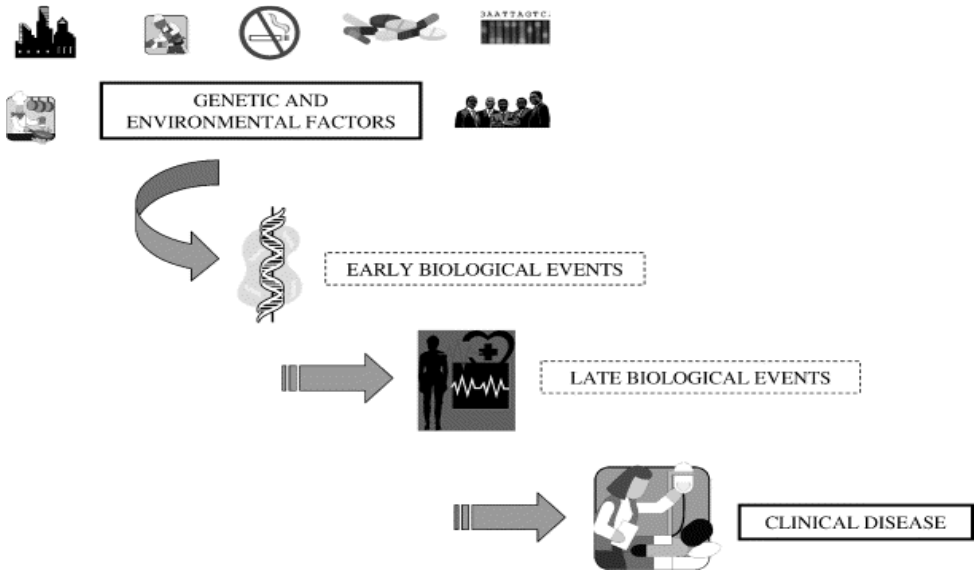

Fig.1 Causal pathway from initiation to the occurrence of disease (Bonassi and Au, 2002).

The methods of molecular epidemiology have a great potential in several areas of cancer research: investigation of etiology of the disease; monitoring of cancer risk in people exposed to occupational or environmental carcinogens; studies of factors protecting against cancer; and assessment of intrinsic factors that might predispose to cancer (Collins, 1998; Albertini et al., 2003a). One application of biomarkers in cancer research, however, has not received

widespread attention – the comparison of background values in normal populations from different regions of the world. Such comparisons, if coupled with incidence of cancers, could lead to the recognition of genetic and/or environmental factors not otherwise appreciated. This naturally requires standardized and validated measurement of biomarkers.

Molecular epidemiology (1) detects various environmental agents or their metabolites in accessible body matrices, (2) measures biological responses to these agents, and (3) defines genotypic or phenotypic factors that modify individual susceptibility. Biomarkers are, therefore, divided into three groups according to whether they reflect the exposure, the effect (early or late), or the susceptibility (National Research Council, 1989; International Programme on Chemical Safety (IPCS), 1993).

An important piece of information gathered for molecular epidemiology is the documentation of exposure. **Biologically active dose** is the amount of the agent that induces detectable effect at the tissue or cellular level. Due to the high sensitivity and specificity, the measurement of chemicals and their metabolites at different stages of their penetration to the body, and the measurement of adducts to macromolecules in body fluids and tissues belong to the main analyzed endpoints. The measurement of adducts is particularly useful for identification of chemicals that form adducts with macromolecules (e.g. protein or DNA adducts) (Shuker, 2002). Among the biomarkers of exposure, DNA and hemoglobin adducts are the best validated biomarkers. Prospective studies clearly demonstrated close relationship between them and the disease process (Qian et al., 1994; Wang et al., 1996). The formation of DNA adducts can lead to DNA replication errors with the potential for carcinogenesis (Baird at el., 2005). For some exposures and genotoxicity endpoints, a quantitative association between exposure and the biomarker has been demonstrated (Albertini et al., 2001; Farmer, 2004).

Exposure to mutagenic chemicals can cause damage to the cellular macromolecules. This damage generally stimulates the affected cells to the response involving, at first, the repair of damage. Health consequences frequently result just from incomplete incorrect repair. Therefore, a variety of biomarkers of early biological effects has been developed and tentatively validated (Norppa, 2004a). The most intensively used biomarkers of early effect are chromosomal aberrations (CA), micronuclei (MN) and hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene mutation. The CA assay is the most extensively used and best validated biomarker of early biological effects in population studies. Cells with chromosomal abnormalities can lead to the development of cancer as indicated by the presence of chromosomal deletions and translocations in the majority of cancer cells

(Mitelman et al., 1997). Use of this biomarker in molecular epidemiology and biomonitoring is the subject of this work and will be discussed later.

The MN assay is an alternative to the CA assay. Instead of detecting the abnormality at metaphase stage of the cell cycle, MN are detected after the cells have passed through the first cell cycle. The major advantage of this assay over the traditional analysis of CA represents the possibility to detect aneuploidy events (Fenech and Morley, 1985; Albertini et al., 2000). In addition, it is much less labor-intensive than the conventional CA assay. On the other hand, the MN assay has also shortcomings. Since the cells need to survive at least one nuclear division, some of the damaged cells may be lost. Although the association between the frequency of MN and long-term biological consequences has not been adequately elucidated yet, a number of indirect evidences support a potential predictive role of this biomarker (Fenech et al., 1999).

The HPRT assay is the most frequently used somatic cell mutation assay in population studies (Perera, 2002; Albertini, 2003b).

Inherited genetic traits co-determine the susceptibility of an individual to a toxic chemical. Individual susceptibility is dependent on several factors such as differences in metabolism, DNA repair, alterations of oncogenes and tumor-supressor genes (Bartsch et al., 1996; Norppa, 2004b). Special emphasis has been put on individual responses to environmental and industrial carcinogens. Molecular epidemiological studies have shown that polymorphic variants of genes involved in the metabolism of carcinogens (Pavanello and Clonfero, 2000; Shields and Harris, 2000) and repair of induced damage (Hu et al., 2002) can act as cancer susceptibility genes. The variants of drug metabolizing and DNA-repair enzymes either increase the activation of chemical carcinogens directly or by diminishing their inactivation or decrease the cells ability to repair mutagenic damage. At the level of human population high frequency of these variants may contribute to a high incidence of cancer cases. For example, "low penetrating" polymorphisms in metabolism genes tend to be much more common in human population than allelic variants of "high penetrating" cancer genes, and, therefore, they are of considerable importance from a public health point of view (Thier, 2003). Mutations in phase I and II enzyme genes involved in xenobiotic metabolism, including genes for CYP1A1, N-acetyltransferase 2 and glutathione-S-transferase (GST) isoforms M1 (null), T1 (null), and P1 (low-activity allele), might confer a low relative cancer risk for an individual. However, because these mutations seem to be common among individuals, they represent a high risk category of genes within population. A growing number of genes encoding enzymes involved in biotransformation of xenobiotics and cellular defense against xenobiotic-induced

damage has been identified and cloned. This led to increased knowledge of allelic variants of genes and genetic defects associated with different susceptibility toward environmental toxicants.

Distinction between particular classes of biomarkers is not always unambiguous, especially between biomarkers of exposure and effect.

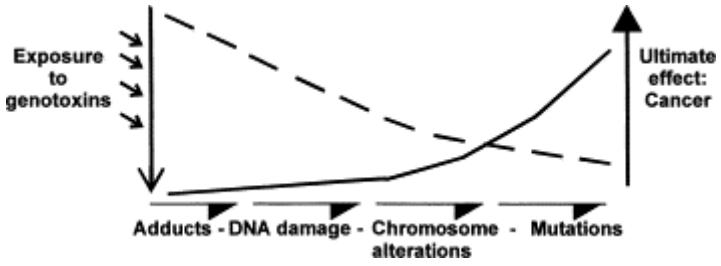

Fig. 2 Biomarkers of exposure and effect. Different markers reflect stages in the sequence of events between exposure and the ultimate endpoint of cancer. Antioxidant protection, metabolic activation/detoxification, DNA repair, and apoptosis operate to modulate the effects of the exposure. As a result, the ability of markers to inform about exposure decreases from left to right (- - -); the ability of markers to predict outcome (or risk) should increase from left to right (–). (From Collins, 1998)

Interpretation of results of biomarker studies usually relates to the value of the measured endpoint. Biomarkers of exposure are usually more chemical-specific than biomarkers of effect. For biomarkers of effect that can be proven to be predictors of disease, a reduction of a biomarker value indicates a reduction of risk. For biomarkers of exposure, reduction of a biomarker value due to an intervention indicates the reduction of exposure, but not necessarily a reduction of risk for all exposed. Inter-individual biological variability affects both, biomarkers of exposure and effect (Binkova et al., 1998; Godderis et al., 2004; Teixeira et.al., 2004). Data are accumulating to support the concept that genotoxicity endpoints are predictors of human cancer risk (Vineis and Perera, 2000; Grant, 2001).

1.2. Chromosomal aberrations as biomarker of exposure or effect

The analysis of cytogenetic end-points as biomarkers of exposure to clastogens has been successfully used in epidemiological studies for many years. The induction of chromosomal aberrations by radiation as well as by chemicals has been studied in various organisms and cells. The first specialized symposium on “Chromosome Breakage” was held in John-Innes

Institute in 1952 (Thomas, 1953). Since then, the microscopic determination of frequency of chromosomal aberrations in PBL (peripheral blood lymphocytes - conventional method of the cytogenetic analysis) has been internationally standardized (Carrano and Natarajan, 1988). Contrary to alternative approaches the process of validation of this biomarker of early effects has been fully accomplished.

Interest in this field has greatly increased during the past few decades in view of the utilization of this phenomenon in mutagenesis testing studies, biological dosimetry in case of radiation accidents, and structure-activity studies of different classes of chemicals. Cytogenetic assays are relatively well characterized as a dosimeter of exposure. The increase in chromosomal aberrations may even be considered a signal of early adverse biological effect. Chromosomal abnormalities are frequently observed in cancer cells, in spontaneously aborted fetuses, and in abnormal newborns.

The cytogenetic analysis of PBL represents the traditional, often applied approach, which has been used to assess the most important occupational and environmental hazards to public health (Bonassi et al., 2005). Human PBL are the cell population of choice for most genetic monitoring studies because they are readily obtainable from a blood sample and are in contact with many body tissues. Lymphocytes circulate throughout the body and can, therefore, be considered to represent systemic exposure in general. The average life span of lymphocytes in human body varies from 2 to 5 years that make them usable for retrospective biodosimetry. The chromosomal aberration assay is primary used for the analysis of structural aberrations which are classified into two main categories: chromosome-type and chromatid-type aberrations.

Chromosome-type aberrations (induced in G_0 lymphocytes by ionizing radiation and radiomimetic chemicals) involve the same locus of both sister chromatids on one or multiple chromosomes. They are generated in G_0 lymphocytes *in vivo* and attain their typical appearance after DNA replication *in vitro*.

Chromatid-type aberrations (induced by most chemical clastogens) affect one or several chromatids of a chromosome or several chromosomes. In cultured lymphocytes they are formed *in vitro* when damaged DNA template is replicated.

The biomonitoring based upon cytogenetic analysis of human PBL has a long tradition in the Czech Republic (Rossner et al.,1995). Thousands of occupationally exposed subjects and control persons have been examined by cytogenetic analysis since 1974. In 1977 National Reference Laboratory of Genetic Toxicology was found. The training of all cytogenetic laboratories in accordance with unified operation protocol (Rossner, 2003), as well as

providing of standard materials and supervision of quality by examination of "blind" sample belonged to the other obligations of National Reference Laboratory. In that way, the excellent conditions for epidemiological research were created, with no analogy in other countries where the cytogenetic analysis was used.

The data on about 12 000 subjects available up to now, represent the biggest database of cytogenetic records. Moreover, all examinations were performed according the standard protocol as was noticed earlier. Here we mention at least the studies from the past few years, e.g. analysis of butadiene exposed workers (Sram et al., 1998), assessment of the spontaneous level of chromosomal aberrations in unexposed adult and child's population of the Czech Republic (Rossner et al., 1998; 2002). Recently, summary experience of the Czech Hygiene Service with cytogenetic analysis has been published (Sram et al., 2004). This work appraises the effects of different types of occupational exposure such as cytostatic drugs, asbestos, coke oven, genotoxins in petrochemical industry, styrene and other hazardous chemicals and correlates them with cytogenetic endpoints.

The system of genetic monitoring helps to detect the population at increased risk of exposure to genotoxic contaminants. The obtained data represent a basis for quantification of exposure and application of preventive action.

1.2.1. Prediction of cancer using chromosomal aberrations

Cytogenetic analysis has been successfully used in occupational medicine for decades as an effective tool for identification of occupational exposure to mutagens and carcinogens. Although some carcinogens are not genotoxic and some *in-vitro* genotoxins are not carcinogenic (Zeiger, 2001), the majority of chemicals classified by the International Agency for Research on Cancer (IARC) as human carcinogens exert also genotoxic effects, including chromosomal aberrations (Waters et al., 1999). This fact provides a strong justification for the use of cytogenetic end-points as biomarkers of the early effects of genotoxic carcinogens.

Nevertheless, at time when the CA in PBL and other cytogenetic endpoints were conceptualized as biomarkers of early effects in the process of carcinogenesis, there was no empirical evidence for causal relationship between incidence of cancers and CA. Not long ago, few epidemiological studies have addressed this issue. Sorsa et al. (1992) reported in a prospective follow-up study that the subjects with a high level of chromosomal aberrations may be at an elevated risk of cancer. Hagmar et al. (1994) and Bonassi et al. (1995) described

a significant increase in the overall mortality in subjects with increased levels of chromosomal aberrations in peripheral lymphocytes.

In the Nordic cohort, the authors observed an increase in total cancer incidence rate [incidence ratio = 2.08; 95% confidence interval (CI), 1.26-3.40] in individuals with the highest CA frequency (Hagmar et al., 1994). In the case of Italian cohort, the mortality ratio for individuals with high frequency of CA in PBL was estimated to be 2.56 (95% CI, 1.35 – 4.86) for total cancer, 4.2 (95% CI, 1.14 – 4.38) for lung cancer, and 4.36 (95% CI, 1.18 – 11.1) for cancer of lymphatic and hematopoetic tissues (Bonassi et al., 1995). When the data from both studies were pooled together, the results suggested that the frequency of chromosomal aberrations in PBL represents a relevant biomarker for cancer risk in humans that reflects either the early biological effects or individual cancer susceptibility, regardless the exposure dose (Hagmar et al., 1998). Similarly, a nested case-control study carried out in a blackfoot endemic area showed statistically significant association between chromosome-type aberrations and cancer (Liou et al., 1999).

Validation of biomarkers as predictors of cancer risk requires to study large populations because investigated events are, in general, rare. Cohort studies on chromosomal aberrations, accomplished by Bonassi et al. (2000), represented an excellent example of such kind of study. In the Czech Republic, the evaluation of CA frequency in PBL has been included in regular medical checkups of workers exposed to selected occupational hazards since 1975. This made feasible identification of a cohort of individuals for prospective follow-up the cancer in order to verify the results of previous studies (Hagmar et al., 1994; Bonassi et al., 1995; Liou et al., 1999). Detailed cytogenetic records in a group of individuals exceeding more than twice the size of the combined Nordic-Italian cohorts allowed to test the hypothesis about predictive character of CA in relation to cancer. The results of cytogenetic analysis of 11 834 subjects who underwent cytogenetic examination for preventive purpose during 1975-2000 were linked to the national cancer registry, revealing a total of 485 cancer cases (Rossner et al., 2005). Significant 24% increase in relative risk (RR) was found in subjects bearing one or more CA. In the analysis of specific cancer sites a significant association between a high level of CA and cancer of intestinal organs was found (RR = 1.86; 95% CI, 1.05 – 3.28), particularly for stomach cancer (RR = 7.79; 95% CI, 1.01 – 60.03). Moreover, significant increase of cancer incidence was described only for chromosome-type aberrations, which are expected to reflect double-strand breaks mostly generated in vivo in G_0 stage, and not for chromatid-type, for which double-strand breaks were probably formed form the initial DNA lesions in vitro in S phase (Pfeiffer et al., 2000).

The relevance of cytogenetic alteration to cancer is supported by the fact that for cancer cells is typical presence of structural and numerical chromosomal aberrations, and micronuclei. These alterations may reflect the genomic instability and clonal evolution of cancer cells rather than immediate effect of genotoxic exposure (Loeb, 2003). Many types of cancer carry specifically balanced chromosomal alterations that are used in the diagnosis of the disease and are considered to have the role in its etiology. These balanced rearrangements appear to act through deregulation of the gene by relocation to an immunoglobulin or T-cell receptor gene, or creation of a hybrid gene by the fusion of parts of two genes (Mitelman, 2000).

The establishment of a correlation between chromosomal aberrations and cancer has stimulated the development of new techniques to detect aberrations in a variety of exposed populations.

1.3. Fluorescence in situ hybridization (FISH)

1.3.1. Application of FISH methods

The technique of *in situ* hybridization was originally developed by Pardue and Gall (1969). At this time radioisotopes were the only labels available for nucleic acids, and autoradiography was the only means of detecting hybridized sequences. However, molecular cloning of nucleic acids and preparation probes with a stable nonradioactive labels removed the major obstacles which hindered the general application of this method. Furthermore, it opened new opportunities for combining different labels in one experiment. Nowadays, unique sequences, chromosomal subregions, or entire genomes can be specifically highlighted in metaphase or interphase cells by **fluorescence *in situ* hybridization** (FISH). This technique can be used to identify chromosomes, detect chromosomal abnormalities or determine the chromosomal location of specific sequences. FISH plays an increasingly important role in a variety of research areas, including cytogenetics, prenatal diagnosis, tumor biology, gene amplification and gene mapping.

While the conventional assay utilizes metaphases only, FISH technique allows the use of both metaphase and interphase cells. Interphase FISH analysis is preferred over conventional cytogenetics in instances where the dividing cells required for chromosome analysis are lacking either because of low mitotic indices or suboptimal metaphase preparation. Even though conventional cytogenetic analysis for screening populations with occupational or

environmental exposure has been relied on in epidemiological studies, the usefulness of such methods is limited because of being restricted primarily to actively dividing cells in culture. Such studies are labor-intensive, require highly skilled personnel, and are prone to technical artifacts during metaphase preparation. In addition, the tandem-labeling FISH approach seems to be sensitive at detecting persistent genetic damage over a longer period of time following exposure to clastogenic agents (Murg, 1999). The ability of FISH to analyze both dividing and non-dividing cells is an important attribute because it reduces sampling errors resulting from using certain subsets of cells (Mark, 1994). Interphase FISH received powerful backing from several recent studies (Parry et al., 1996; El-Zein et al., 2001; Nordgren, 2003; Zemanova et al., 2005).

Doak et al. (2004) described a method for designing and producing locus-specific interphase FISH probes for any human genomic region. Centromeric, telomeric and locus DNA-sequence specific probes can be used to identify aneuploidy or gene mutations. Several protocols combine molecular karyotyping with classic cytogenetics (Zamo et al., 2006). Other sophisticated, FISH-based protocols have been introduced. Among them, comparative genomic hybridization (CGH) is very important because it can detect non-balanced chromosomal aberrations in uncultured tumor cells (Kontogeorgos, 2004). This method was initially developed for comparison of two genomes at the metaphase chromosome level. This approach allows rapid detection of DNA amplification or loss anywhere in the tumor genome and maps these changes. CGH is based on competitive *in situ* hybridization of different labeled tumor DNA and normal DNA to human metaphase chromosome preparations. Gains or loss of chromosome sequences can be observed as an altered ratio of the two fluorochromes used to detect the normal or tumor DNA specifically.

More recently, CGH has been conducted using genomic arrays as an alternative to metaphase preparations. The microarrays are constructed from large insert clones and are used to identify chromosomal imbalance (i.e., duplications, amplifications, and deletions). Comparative hybridization using tumor and normal DNA samples labeled with different fluorochromes is similar to that for any two-dye competitive hybridization microarray method. A recent example of the use of microarray-based CGH is provided by Hurst et al. (2004). A review of the current status and future prospects of array-based CGH is presented by Snijders et al. (2003).

The combination of different FISH methods represents very effective approach. For example, spectral karyotyping (SKY) and interphase FISH showed that 45% of cases with normal G-banded karyotypes actually contained a variety of different chromosomal changes

in the malignant clone. Several prognostically important aberrations were detected that would have been overlooked using conventional banding techniques alone (Nordgren, 2003).

1.3.2. Whole-chromosome painting - principle of method

The detection of translocations is becoming more feasible with the development of fluorescence *in situ* hybridization with DNA probes that paint specific chromosome pairs along their entire lengths. This method allows the selective identification of each pair of chromosomes in human genome and permits rapid recognition of chromosomal breakage and exchange events between painted and non-painted chromosomes.

The basic principle of the method is that single-stranded DNA will bind or anneal to its complementary DNA sequence. Thus, a DNA probe for a specific chromosome will recognize and hybridize to its complementary sequence on a metaphase chromosome or within. Both have to be in a single-strand conformation, therefore the DNA probe and the target DNA must be denatured, usually by heating in a formamide-containing solution. The probe is hybridized to the target DNA under conditions that allow the DNA to reanneal in double-strand form. Whole chromosome paints are cocktails of sequence probes that recognize the unique sequences spanning the length of a particular chromosome. At metaphase, both chromosome homologues are "painted" or emit fluorescence light. To detect the probe on the target DNA, the probe DNA can be directly labeled with a fluorescent tag. It can also be chemically modified by the addition of hapten molecules (biotin or digoxigenin) that can then be indirectly fluorescently labeled with immunocytochemical techniques. The target DNA is counterstained with another fluorochrome of a complementary color.

FISH painting is predominantly applied to chromosome-type aberrations, using the quantitative assessment, where the data are expressed as “aberrations per cell” and “breaks per cell”. In case of primary aberration, it provides easy detection of some symmetrical forms, like reciprocal translocations, not readily visible with conventional staining, and only scored with difficulty when chromosome banding techniques are applied. Since these symmetrical forms are the ones most likely to be transmitted to future cell generations (as “secondary” or “derived” chromosome-type aberrations), FISH painting has proved ideal for long-term follow up studies after radiation exposure (Straume et al., 1991; Lucas et al., 1992) and also for clinical and cancer characterization. Nacheva et al. (2000) demonstrated that the use of painting is a suitable tool for routine diagnostics in screening patients for a particular pattern

of chromosome involvement. This is especially true when the karyotypic analysis is difficult (for example in patients under treatment or after bone marrow transplantation) and when the highly sensitive methods such as RT- PCR are restricted by the absence of specific disease markers.

The results of several studies indicate that scoring of symmetrical translocations using FISH provides an alternative biodosimetric approach to conventional dicentric analysis for a retrospective assessment of the radiation dose. Translocations frequencies, unlike unstable dicentrics, do not essentially change with time after exposure and their visualization by chromosome painting is suitable method for assessing past or chronic exposures (Salassidis et al., 1994&1998; Stephan et al., 2000; Lindholm, 2001). For example, Lindholm et al. (2002) calculated that dicentrics decreased rapidly with half-time of approximately 2 years, whereas the frequency of all translocations fell on average to about 65% of their initial value during 4 years. Furthermore, two-way translocations were slightly more persistent than other translocations. The average half-time was about 8 years for two-way translocations and around 6 years for other translocations. It had been postulated by Natarajan et al. (1996) that formation of translocations in comparison with dicentrics has not been affected by the condensation state of nuclei but different types of repairable lesions and repair kinetics were involved in formation of dicentrics and translocations. In addition, Bassi et al. (2003) pointed out preferential elimination of unstable aberration by p53-dependent apoptosis through generation of changes in mitotic spindle. These findings suggest that translocations could represent a better biodosimetry tool than dicentrics. In case of radiation dosimetry it is important that all estimates can be influenced by the received radiation doses and the health status of the individuals studied.

FISH with whole chromosome paints has been the primary method of choice for most investigators who wished to quantify and characterize chromosome damage from environmental or occupational exposure. The reasons for using FISH painting have been the speed of assay and the ability to detect relatively stable events such as translocations in parallel with the enumeration of unstable dicentrics. This combination of features has enabled investigators to evaluate recent exposures as well as those that occurred many years ago and to characterize lower level of exposure than were possible with older cytogenetic methods. Due to its widespread success few investigators now debate the utility of chromosome painting for assessing exposure to genotoxic agents. As the field of molecular cytogenetic matures, it is appropriate to determine what must be done to improve our ability to evaluate cytogenetic risks associated with environmental exposure in human populations.

Currently majority of painting is performed with the small number of probes at a time, usually with just one color of paint, but sometimes with two or three chromosomes labeled in different colors. Each additional probe in the cocktail increases the proportion of the genome, in which aberration can be observed, and also increases the fraction of all exchanges, that can be detected. However, there are additional costs associated with purchasing each paints in the cocktail as well as concomitant increase in the analysis time for each cell. Some years ago, two groups reported the ability to paint each of the 24 human chromosomes in a unique color, thereby enabling the identification of every interchromosomal exchange in each cell. Spectral karyotyping (SKY) (Schrock et al., 1996) and multicolor FISH (mFISH) (Speicher et al., 1996) are currently enjoying widespread use in clinical cytogenetics (Tchinda et al., 2004; Aamot et al., 2005). Although research application have been more limited, mFISH and SKY have been successfully employed to evaluate chromosomal rearrangements in individuals occupationally exposed to internal plutonium (Anderson et al., 2005) and as biodosimetry tool after gamma irradiation (Szeles et al., 2006). Unfortunately, this approach requires expensive probes and the analysis time per cell is substantially longer than when only a few chromosomes are painted. As a result, the technology of painting every chromosome in its own color has not been applied frequently to the evaluation of human population.

In the field of molecular epidemiology, FISH painting has been applied regretfully rarely. Rubes et al. (1998) studied genotoxic effect of occupational exposure to cytostatics in medical staff by painting chromosomes #1 and #4. They found significantly higher number of translocations in exposed than in control individuals ($F_G/100 = 2.25\pm1.50$ vs. 0.66 ± 0.21, $p<0.01$). Moreover, the difference between analyzed groups was more pronounced in comparison with conventional cytogenetic analysis – FISH thus appeared to be more sensitive than classical approach.

Zhang et al. (2005) used three-color painting to detect specific aneuploidy among all 24 chromosomes in benzene-exposed workers. Their results suggest that benzene has the capability of producing selective effects on certain chromosomes. Among analyzed chromosomes, monosomy of chromosomes #5, #6, #7, and #10 had the highest incidence ratios ($IR>2.5$, $p<0.01$).

Painting of chromosome #4 was used by Maeng et al. (2004) to evaluate DNA damage in chromium-exposed workers. A significant positive correlation between the translocations detected by FISH and the content of Cr in the blood was found.

Stable aberrations measured by FISH painting were established among Finnish nuclear power plant workers by Lindholm (2001). Although the mean cumulative dose was only

moderate (approximately 100 mSv), this approach allowed to detect the effects of such exposure to ionizing radiation.

The suitability of three-color painting was approbated by Verdorfer et al. (2001) in five different group of people: 30 employed in radiology, 26 employed in nuclear medicine or radiation physics, 32 patients with breast cancer, 26 handling military waste disposal, all presumably exposed to low doses of radiation or chemical mutagens and a non-exposed control group (N=29). Increase in the frequency of translocations measured by FISH (chromosome #2 and #4) was seen in tumor patients and radiation appliers, while a higher proportion of unstable aberrations was found in the chemically exposed individuals. Moreover, chromosome #4 was declared to be more sensitive than chromosome #2.

Tucker et al. (2003) studied effect of occupational exposure to phosphine. In this case, FISH painting did not reveal any increase in the level of traslocations.

The main problem of such studies is the number of people involved in experiment due to expensiveness and laboriousness. Our database of cytogenetic outcomes measured by three-color painting for chromosomes #1 a #4 is unique in many ways. It consists of about 900 healthy people (some of them were analyzed more then once) from which about 500 were occupationally exposed to the scale of clastogens such as polycyclic aromatic hydrocarbons (PAHs), acrylonitrile (ACN), ethyl benzene (EB) or irradiation in nuclear power plants.

1.4. Cytogenetic endpoints and genetic polymorphisms

During the last few years, increasing attention has been focused on genetic polymorphism that could modulate human response to genotoxic damage (Norppa, 2003; De Roos et al., 2004). Theoretically, any polymorphism that affects xenobiotic metabolism or cellular response to DNA damage (genes that control cell differentiation, cell cycle kinetics, signal transduction and DNA repair) could alter individual sensitivity to genotoxins. The best way to study the role of genetic polymorphism is to study the effect of exposure using biomarkers such as cytogenetic endpoints. In contrast to study an incidence of a particular genotype among cancer patients, the information about exposure is usually available and, in addition, there is the possibility to compare the level of biomarker in groups with different genotype.

Several studies have implied that genetic polymorphism can influence not only the level of chromosomal damage associated with some genotoxic exposures, but also the background level of cytogenetic alterations (Sram et al., 1998; Norppa, 2004b; Teixeira et al., 2004;

Vodicka et al., 2004; Georgiadis et al., 2005; Mateuca et al., 2005). Thus, genetic polymorphism might partly explain the association between levels of chromosomal aberrations and cancer risk.

Inter-individual variability in xenobiotic metabolism has been attributed in part to the polymorphic expression of several phase I activation and phase II detoxification enzymes. The role of these genetic polymorphisms in susceptibility has been most extensively evaluated for isozymes of cytochrome P450 (CYP1A1, CYP2D6, and CYP2E1), N-acetyltransferase (NAT1 and NAT2), glutathione S-transferase (GSTM1, GSTT1, and GSTP1), and microsomal epoxide hydrolase (Clapper, 2000). These genetic polymorphisms may together influence genotoxicity of clastogens (Huang et al., 1999; Salama et al., 2001). Following paragraphs summarize the results of several recent studies focused to the effects of polymorphisms in these genes.

P450 cytochromes are enzymes which catalyze the insertion of one atom of molecular oxygen into substrate. This is a typical reaction of activation (phase I) which converts indirect carcinogens (e.g., PAHs, nitrosoamines, some components of tobacco smoke and many solvents, including benzene) into active electrophiles capable of interaction with the biological macromolecules. Among 194 non-smoking subjects exposed to PAHs, carriers of at least one CYP1A1*2A allele had increased level of cells with chromosomal aberrations (mean personal exposure to benzo[a]pyrene <0.9 ng/m^3 during the previous 24h) in comparison with individuals carrying only wild type of allele (Georgiadis et al., 2005). A significant association between the CYP1A1 MspI polymorphism and frequency of chromosomal aberrations was observed in the newborns ($p=0.02$), with heterozygotes showing higher aberration frequencies than the wild type homozygotes (Pluth et al., 2000a).

Glutathione S-transferases (GSTs) are the important group of detoxifying enzymes of phase II. This family of enzymes is responsible for the glutathione conjugation and metabolic detoxification of various reactive species of many chemicals, including the diol epoxide of benzo[a]pyrene. Null genotypes for the GSTT1 and GSTM1 genes have been identified to be associated with an increase of cancer risk in relation to smoking (Strange and Fryer, 1999). Studies on cytogenetic biomarkers have supported the idea that GSTM1 null individuals have a reduced capacity to detoxify genotoxins in tobacco smoke. GSTM1 null subjects had increased frequency of CA (Scarpato et al., 1997; Norppa, 2001; Tuimala et al., 2004). Bus drivers with the GSTM1 null and slow acetylator NAT2 genotype had an increased frequency of cells with chromosomal aberrations (Knudsen et al., 1999). On the other hand, in group exposed to 1,3-butadiene (0.53 mg/m^3) GSTM1 deficiency was closely connected with lower

frequency of chromosomal aberrations (Sram et al., 1998). This effect of GST polymorphisms was proved in several studies. For example, GSTT1-null genotype was associated with decrease in micronuclei (MN) frequency (Yong et al., 2001; Leopardi et al., 2003). In smoking shoe workers, an effect of the occupational exposure on the frequency of micronucleated cells could be seen only in GSTM1 null subjects (Pitarque et al., 2002).

N-Acetyltrasferase 2 (NAT2) metabolise such genotoxins as aromatic amines found in cigarette smoke. High baseline CA level has been associated with the NAT2 slow acetylator genotype (Knudsen et al., 1999; Norppa, 2001; Pluth et al., 2000b (in non-smokers)). Among pesticide-exposed greenhouse workers and controls, however, an increased frequency of MN was found in smokers with NAT2 rapid acetylator (Falck et al., 1999). Similarly, Pluth et al. (2000b) observed that heavy smokers with NAT2 rapid acetylator genotype have a higher frequency of translocations than those with the slow acetylator genotype.

Microsomal epoxide hydrolase (mEH) is another phase II metabolising enzyme, which catalyse the hydrolysis of epoxides into dihydrodiols. This reaction usually leads to detoxification, but in case of PAHs, mEH represents a part of metabolic activation route generating highly reactive diolepoxides. The human mEH gene (EPHX1) is polymorphic. Base substitutions resulting in amino acid change are found in exon 3 and 4 and lead to 25-39% reduction and 24-25% increase in EPHX1 activity, respectively. On the basis of exone 3 and 4 genotypes the expected EPHX1 activity can be classified as low, intermediate, and high. It is assumed that high activity EPHX1 genotype confers increased sensitivity to PAHs in tobacco smoke. In agreement with this theory, smoking lung cancer patients with high or intermediate activity of EPHX1 had a higher frequency of chromosome #1 breakage (tandem probe assay) in comparison with smoking patients with low activity of EPHX1. Such effect was not, however, observed in healthy control smokers from the same study (Cajas-Salazar et al., 2003). Another study indicated that EPHX1 exon 3 genotype could affect MN frequency in PAH-exposed potroom workers, but the carriers of exon 4 variant allele did not show similar correlation (Carstensen et al., 1999). High activity EPHX1 genotype correlated with a moderate decrease of CA in the unexposed subjects (Migliore et al., 2006) while low activity EPHX1 genotype increased CA in styrene exposed workers (Vodicka et al., 2001).

The X-ray cross-complementing gene 1 (XRCC1) is involved in base-excision repair (Thompson and West, 2000). Carriers of the XRCC1 codon 280His variant allele had a two-fold level of unstable exchanges (dicentrics and ring chromosomes) (Kiuru et al., 2005). The frequencies of total MN were borderline associated with the Arg(194)Trp polymorphism but not associated with the Arg(280)His, Arg(399)Gln and Gln(632)Gln polymorphisms among

coke-oven workers (Leng et al., 2005). Individuals from unexposed population carrying variant alleles for XRCC1 codons 280 and 194 showed a decreased level of chromosome-type breaks. The effect of XRCC1 codon 399 genotype on the frequency of CA was modified by smoking (Tuimala et al., 2004).

The xeroderma pigmentosum group D (XPD) protein is involved in the nucleotide excision repair (NER) pathway, which recognizes and repairs a wide range of structurally unrelated lesions such as bulky adducts and thymidine dimers (De Laat et al., 1999). Several epidemiological studies have investigated the association between XPD polymorphism and cancer (Benhamou and Sarasin, 2002; Matullo et al., 2005). Previously, the XPD 751 Gln and 312Asn variant alleles were associated with a decrease of dicentrics induced by X-rays and an increase of chromatid breaks and aberrant cells induced by UV in human lymphocytes *in vitro* (Au et al., 2003). The Gln/Gln genotype has been linked to decrease of total CA frequencies, particularly among smokers (Vodicka et al., 2004). Affatato et al. (2004) observed a significantly increased frequency of spontaneous and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NKK)-induced chromosomal breakage in smokers and subjects > 48 years old carrying the codon 312Asn variant alleles; the effect of codone 751Gln allele was not significant. The study of Kiuru et al. (2005) indicated no significant changes in the amount of CA that could be attributed to the XPD codon 751 polymorphism.

The human 8-oxo-guanine DNA glycosylase I (hOGG1) gene encodes a DNA glycosylase/AP lyase that is involved in the BER (base excision repair) of 8-hydroxyguanine from double stranded DNA (Shinmura and Yokota, 2001). The best known polymorphism in the hOGG1 gene is the Ser/Cys polymorphism at codon 326, and the 326Cys variant allele has constantly been associated with an increased risk of various type of cancer (Goode et al., 2002). Kiuru et al. (2005) observed no association between the hOGG1 codon 326 polymorphism and any of the CA studied. However, previous reports had shown that the OGG1 326Cys allele had a lower ability than the OGG1 326Ser allele to prevent mutagenesis induced by 8-hydroxyguanine in human cells *in vivo* (Yamane et al., 2004) and is associated with a reduced DNA repair capacity for DNA oxidative damage (Aka et al., 2004).

Folate provides methyl group for DNA methylation and the conversion of uracil to thymidine. Therefore, folate deficiency can disrupt global DNA methylation patterns (Jacob et al., 1998) and induces excessive incorporation of uracil into DNA (Blount et al., 1997). Simultaneous removal of two uracil bases on opposite DNA strands within 12 bp may result in the formation of double strand break (Frosst et al., 1995) and subsequent chromosomal breakage (Fenech, 2001). Homozygous TT variant genotypes (C677T polymorphism in

methylenetetrahydrofolate reductase (MTHFR) gene) have *in vitro* about 30% of normal enzyme activity, and heterozygous individuals have 65% enzyme activity. This polymorphism affected frequency of MN in cultured human lymphocytes: the TT cells showed about 20% higher levels of MN than CC cells (Kimura et al., 2004). On the other hand, no effect on MN was observed in lymphocytes of traffic wardens (Zijno et al., 2003). Methionine synthase (MS) catalyzes the transfer of methyl base from 5-methyltetrahydrofolate to homocysteine. It is reported to have a polymorphism in 2756A to G (glycine→aspartic acid), resulting in lower enzyme activity, homocysteine elevation and DNA hypomethylation (Leclerk et al., 1996).

2. Aims of study

In spite of proved advantages in detecting and identifying of chromosomal changes in comparison with conventional cytogenetic analysis FISH painting have been used regretfully rarely to evaluate genetic risk associated with environmental exposure in human populations. Therefore the aims of this study were as follows:

1) to verify suitability of FISH painting to evaluate genotoxic effects of different environmental clastogenes such as carcinogenic polycyclic aromatic hydrocarbons (c-PAHs), acrylonitrile, ethyl benzene, butadiene or irradiation in nuclear power plants;

2) to compare effectiveness of two cytogenetic approaches-FISH painting and conventional cytogenetic analysis on the same occupationally exposed groups and matched controls;

3) to establish a possible connection between other biomarkers and chromosomal aberrations;

4) to follow the spectrum of chromosomal damage and possible individual susceptibility of painted chromosomes in response to different genotoxins.

3. Results (see Appendixes 1-5)

Appendix 1:

R.J. Sram, **O. Beskid**, B. Binkova, P. Rossner, Z. Smerhovsky: Cytogenetic analysis using fluorescence in situ hybridisation (FISH) to evaluate occupational exposure to carcinogens.

Toxicology Letters 149 (2004) 335-344.

Appendix 2:

O. Beskid, B. Binkova, Z. Dusek, P. Rossner, I. Kalina, T.A. Popov, P.B. Farmer, R.J. Sram: Chromosomal aberrations by fluorescence in situ hybridisation (FISH) – biomarker of exposure to carcinogenic PAHs.

Mutation Research 2006, submitted, 25 p.

Appendix 3:

R.J. Sram, **O. Beskid**, B. Binkova, I. Chvatalova, Z. Lnenickova, A. Milcova, I. Solansky, H. Bavorova, D. Ocadlikova, P.B. Farmer: Chromosomal aberrations in environmentally exposed population in relation to metabolic and DNA repair genes polymorphisms.

Mutation Research, submitted, 47 p.

Appendix 4:

R.J. Sram, P. Rossner, J. Rubes, **O. Beskid**, Z. Dusek, I. Chvatalova, J. Schmuczerova, A. Milcova, I. Solansky, H. Bavorova, D. Ocadlikova, O. Kopecna, P. Musilova: Possible genetic damage in the Czech nuclear power plant workers.

Mutation Research 593 (2006) 50-63.

Appendix 5:

O. Beskid, Z. Dusek, I. Solansky, R.J. Sram: The effects of exposure to different clastogens on the pattern of chromosomal aberrations detected by FISH whole chromosome painting in occupationally exposed individuals.

Mutation Research 594 (2006) 20-29.

4. Discussion

In all our studies we analyzed chromosomal aberrations by FISH in total in about 900 subjects. It seems to be the largest set of data using whole chromosome painting with chromosomes #1 and #4. Therefore we believe we can use our results to generalize what we can expect from the method used.

4.1. Cytogenetic analysis by FISH to evaluate occupational exposure to carcinogens (Appendix 1).

We attempted to compare the ability of conventional cytogenetic analysis and FISH technique with the whole chromosome painting for chromosome #1 and #4 to detect chromosomal damage in occupationally exposed populations. In this context, the advantages of the FISH techniques, such as better sensitivity, reliability and objectivity are usually discussed. However, practical experiences with the use of these techniques for biomonitoring of clastogenic exposures are limited. Therefore, we applied both methods on the same occupationally exposed groups and matched controls to see the differences in results. First of all we interested in the sensitivity of these methods under conditions common in public health practice.

Results showed significant clastogenic activity of ethyl benzene and benzene. This suggestion became a background for imposing strict preventive measures, the use of personal protective equipment during each process, when workers are exposed to benzene and ethyl benzene. Substantial decrease of observed translocations after 10 months may be interpreted as a successful health intervention.

In the group occupationally exposed to ethyl benzene, the decrease of translocations seems to be more pronounced than the decrease of chromosomal breaks by conventional method. Usually, it is expected that stable translocations should circulate in lymphocytes longer than unstable chromosomal breaks. Hoffmann et al. (1999) evaluated the decline in cells bearing translocations to be approximately 13% per division. The information about exposure to carcinogens and persistence of translocations detected by FISH are lacking and our data represent a first contribution to this topic. They indicate, that substantial decrease in personal exposure to ethyl benzene and benzene in the course of more than 6 months can also decrease the level of stable translocations determined in peripheral lymphocytes. We can hypothesize that cells carrying several translocations do not circulate for years, but may be

also eliminated as cells carrying chromosomal breaks. It seems to be pertinent to use FISH analysis repeatedly on the same subjects, trying to specify the relationship between the exposure and effect (expressed as stable translocations).

In spite of the fact that most of features make the FISH superior to the conventional method, there is one circumstance, when an interpretation of results obtained by FISH and conventional method may differ. The FISH detects translocations, which are long lasting injuries likely transferred through many cell cycles. It means that the FISH measures the type of chromosomal changes related to cancer and that these changes may circulate in PBL for a long period of time. However, we have observed the decrease in the level of chromosomal aberrations determined by the FISH within the period of ten months. It seems that it will be possible to use this method in the similar way as the conventional analysis not only to detect the exposure to clastogens but also to check an efficiency of improved working conditions.

4.2. Impact of environmental exposure to carcinogenic PAHs (Appendix 2 and 3).

Under the EXPAH project we were able to study the impact of environmental exposure to c-PAHs in Prague, Kosice and Sofia *(Appendix 2)*. The basic difference between the studied exposed groups (police officers, bus drivers) and controls is that the police officers usually walk through busy streets in 8-12 h shifts, but controls spent more than 90% of their time indoors. Usually it is calculated that in a non-smoking house the concentration of PM2.5 and c-PAHs is 50-60 % of outdoor pollution. It means that the police officers may be exposed to approximately twice the concentration of PM2.5 and c-PAHs than the controls during their work shifts. In all three cities the level of air pollution to c-PAHs significantly increased the genomic frequency of translocations during the winter period.

This was the first study when the FISH method was used to analyze the impact of environmental air pollution. According to the original hypothesis it was expected that the most important group of chemicals responsible for the biological activity of air pollution represent c-PAHs. Using biomarkers of exposure, an increased level of DNA adducts in exposed groups compared to controls has usually been observed (Peluso et al., 1998). In contrast, using cytogenetic endpoints as biomarkers of effect, no increase of chromosomal aberrations by the conventional method, sister chromatid exchanges or micronuclei by air pollutants was observed in Europe (Binkova et al., 1996; Bolognesi et al., 1997 a,b).

The obtained results indicate a significant genotoxicity of organic compounds adsorbed onto ambient air particles, which induce stable translocations determined in peripheral lymphocytes. It corresponds to genotoxicity of extracted organic matter from air particles as

was proved by Binkova et al. (2003) for the sample from Prague. We may conclude that FISH analysis indicates that police officers in Prague, Kosice and Sofia as well as bus drivers in Sofia represent a group with increased genotoxic risk. We may postulate that the FISH method seems to be more sensitive than other cytogenetic endpoints, and it may be concluded as well that the increase of genomic translocations represent a more significant health risk for their carriers, especially for the process of carcinogenesis.

Different factors, which could affect genomic frequency of translocations as life style, antioxidant vitamin levels or genotypes of metabolic genes were studied specifically in Prague *(Appendix 3)*.

Using conventional cytogenetic analysis, no differences in the frequency of chromosomal aberrations were observed between the city policemen working in winter outdoors in the Prague downtown area and controls. The level of chromosomal aberrations in exposed and control groups corresponded to the spontaneous frequency of chromosomal aberrations in the Czech Republic (Sram et al., submitted).

Using a whole chromosome painting for chromosomes #1 and #4, the frequency of chromosomal aberrations, detected mostly as translocations, was significantly higher in city policemen compared to controls. This effect of city air pollution was also observed by comparing subgroups of exposed nonsmokers vs. control nonsmokers. The effect of smoking was especially significant among controls.

Multiple regression analysis for FISH, when we studied the relationship between chromosomal aberrations and DNA adducts in the same subjects (Binkova et al., submitted), indicated that B[a]P-like DNA adducts became a significant predictor of genomic frequency of translocations, percentage of aberrant cells, as well as aberrations per cell.

The observed effect of air pollution during the winter season in downtown of Prague indicates that exposure to c-PAHs may represent a significant health risk for exposed policemen. We may speculate that a long term exposure to ambient air concentrations of c-PAHs 20-25 ng/m3, B[a]P 2.9-3.5 ng/m3 (Binkova et al., 2003) may already damage DNA, inducing DNA adducts which remain unrepaired and finally are manifested as structural chromosomal aberrations – translocations.

The fact that ambient air concentrations of c-PAHs induce simultaneously DNA adducts and structural chromosomal changes seem to be of high importance. It means the increased risk of cancer and atherosclerosis (De Flora et al., 1997; Binkova et al., 2002). Despite the fact that air pollution is only one factor among others (as stress or life style), it might be

concluded that city policemen in downtown of Prague are at higher risk of cardiovascular diseases. The obtained knowledge should be used for prevention.

The effect of exposure in city policemen, detected as chromosomal aberrations, was further modulated by genetic polymorphisms – by conventional method for metabolic gene CYP1A1*2C and DNA repair genes XPD exon 6 and exon 23, by FISH method only for metabolic genes CYP1A1*2C, EPHX1, GSTP1, p53 MspI, MTHFR677. Polymorphism of metabolic and DNA repair genes identify subjects susceptible to DNA damage by exposure to carcinogenic PAHs.

The interpretation of the significance of genetic polymorphisms and chromosomal aberrations, to explain its significance for cancer risk, seems to be a very difficult task. Chromosomal aberrations are a biomarker indicating an increased risk for health consequences. We observed in high activity genotype EPHX1 carriers decreased frequency of genomic frequency of translocations. Similarly Vodicka et al. (2004) found a lower frequency of cells with aberrations in subjects with the same genotype using conventional cytogenetic analysis.

We may speculate that cancer susceptibility of individual subject is enhanced by deficiency in the detoxification of toxic metabolites formed from air pollutants. Mutant variants of DNA repair genes increase the susceptibility of their carriers. Chromosomal aberrations may be understood as a complex biomarker, reflecting the exposure to carcinogens as well as their effects on our genetic background.

Ambient air exposure to c-PAHs significantly increased FISH cytogenetic parameters in nonsmoking policemen from the city center of Prague. Results of FISH analysis indicate that city policemen represent a group of the increased genotoxic risk. It was for the first time when the relationship between DNA adducts (biomarker of exposure) and chromosomal aberrations by FISH (biomarker of effect) was observed.

4.3. Genetic damage in nuclear power plants (Appendix 4).

In our study, cytogenetic endpoints from 273 subjects were analyzed by the conventional cytogenetic analysis and 286 subjects by FISH

Workers from two nuclear power plants (NPP) participated; the Dukovany nuclear power plant has been in operation for almost 20 years, the new Temelin nuclear power plant for 2 years. We did not see any increase of chromosomal aberrations related to the occupational radiation exposure in either nuclear power plant. It was neither found by the conventional cytogenetic analysis nor – surprisingly – the more sensitive FISH method.

No effect of irradiation was observed on the induction of genetic damage in peripheral lymphocytes, when short and long term exposure to radiation was related to the frequency of chromosomal aberrations by the conventional cytogenetic analysis and translocations by FISH at the Temelin nuclear power plant as well as the Dukovany nuclear power plant.

We did not observe any relationship between the frequency of chromosomal aberrations and radiation dose. We may attempt to explain our results by very low doses of radiation which were detected as the long-term exposure in the Czech nuclear power plants. We have examined recently published papers to find a possible dose of radiation inducing clastogenicity in nuclear power plants: Germany – 390 mSv (Braselmann et al., 1994), Finland – 100 mSv (Lindholm, 2001), Russia 0-8.5 Gy (Burak et al., 2001), Bulgaria – 200 mSv (Hadjidekova et al., 2003), Great Britain – 50-500 mSv (Tawn et al., 2003; Tawn et al., 2004), Belgium – 16 mSv (Aka et al., 2004). We may therefore speculate that if the level of ionizing radiation remains in the next years at the same level as before the year 2002 (when we collected samples), then we should not expect to see any occupational genetic damage in workers going to the "hot" (monitored) zone. Simultaneously, we may postulate that preventive safety measures in the Czech nuclear power plants will maintain this low level of occupational exposure to radiation.

The occupational level of ionizing radiation at the Czech nuclear power plants is really very low – (beneath the average annual background radiation of 1.0 mSv, the public dose limit mandated by ICRP) (ICRP, 1991).

Such low ionizing radiation exposure may induce adaptive response. A previous in vivo exposure may have an effect on the individual chromosomal radiosensitivity as a result of an adaptive response phenomenon. According to Thierens et al. (2002), short-term low-dose occupational exposure < 10 mSv may act as an in vivo adaptive dose and stimulate repair in G_0 lymphocytes. But the dose at the Czech nuclear power plants may be too low to induce adaptive response. We may postulate that the doses of radiation were so low, that they were not able to increase the frequency of chromosomal aberrations determined by either way.

We have only observed that the effect of lifestyle and genetic susceptibility factors on the chromosomal damage reflects the exposure to chemical clastogens (which corresponded to our previous results; Sram et al., 2004) rather than ionizing radiation. The genomic frequency of translocations was affected by smoking, alcohol consumption, chronic use of drugs, and genetic polymorphisms of genes GSTP1, and p53 Bst.

Finally, we may conclude that low doses of ionizing radiation to which workers in the Czech nuclear power plants are exposed do not represent by themselves any significant risk of genetic damage as measured by the conventional cytogenetic analysis and FISH.

4.4. Specificity of chromosomal aberrations with a different exposure (Appendix 5).

In another study, we attempted to analyze the effects of different kinds of clastogen exposure with FISH cytogenetic endpoints determined by the whole chromosome painting. The study tried to answer the question whether or not any specificity exists in the type of chromosomal aberration in any of both painted chromosomes.

Our studied groups were only moderately affected by ACN, c-PAHs, or irradiation at NPP. Significant changes were detected solely in the group of EB production. It corresponded to the idea that clastogenic effect depends on the concentration of carcinogen in a working environment as well as the use of preventive measures (Sram et al., 2004).

Nevertheless, observed changes between control and clastogen exposed groups seemed to have a distinct pattern: the percentage of translocations was relatively lower in the EB group, but of reciprocal translocations in ACN and NPP-exposed groups, respectively.

In the chemical clastogens exposed groups (ACN, EB, c-PAHs), there was also a significant increase in the relative number of insertions. Nevertheless, we cannot fully exclude the possibility that this difference is rather a consequence of small absolute numbers of insertions and that they lack any meaningful biological background – for example, in the control group there were no insertions at all. More detailed analysis of the effects of aging on the aberration pattern is consistent with the following principle: the observed increase took place in the proportion of translocations and reciprocal translocations which are highly stable structures because they do not obstruct chromosomal disjunction or cause a loss of genetic information during mitosis (Pressl and Stephan, 1998). On the other hand, the relative number of acentric fragments did decrease with time: the lack of a centromere in these fragments can lead to impaired distribution of genetic material during cell division and subsequent elimination of such affected cells.

We found out that smoking had no effect on the aberration pattern, i.e. smoking does not induce preferentially any specific type of chromosomal aberration.

Chromosomes #1 and #4 differ in physical length: □#1 represents 8.28% of human genome in males, whereas #□4 represents 6.39%. Physical length for each chromosome was calculated according to DNA content adjusting to male genome (Morton, 1991). The ratio of observed aberrations on chromosomes #1 and #4 in the control group corresponds to the ratio

of their size, i.e. approximately 1.3. However, all types of exposure exhibited higher ratios. Therefore, we decided to relate the proportion of cells with aberration on chromosomes #1 and #4, respectively, obtained in different exposure groups, to the control levels.

As far as the relative numbers of cells with aberration on chromosomes #1 and #4 are concerned, the multiple regression analysis indicated a decrease of aberrations on chromosome #1 but an increase on chromosome #4 in the groups exposed to ACN and EB. A similar trend may be observed also for c-PAHs and NPP-exposed groups. Moreover, chromosome #1 seemed to be slightly more sensitive to the adverse impact of smoking than chromosome #4, which is contrary to the previous idea on the effect of c-PAHs.

It can be hypothesized that an unequal distribution of aberrations on individual chromosomes can be attributed to an uneven distribution of repair genes and/or genes regulating the cell cycle on the chromosomes and that these genes can be specifically activated or deactivated by the exposure agents or their metabolites. The results of Surralles et al. (1998) indicated that the level of excision repair synthesis is higher in high gene density chromosomes (chromosome #1 in our case) and, therefore, that these chromosomes are preferentially repaired. Moreover, transcription-coupled repair, a specialized nucleotide excision repair pathway, was shown to be organized in clusters incorporating predominantly early-replicating gene-rich bands within the human genome (Surralles et al., 2002). Chromosome #4 is known to be gene-poor and late-replicating whereas chromosome #1 had more chance for being repaired. Also Puerto et al. (1999) verified that chromosome with high gene density are preferentially repaired in human cells.

Our analysis was done on a total sample of 319 subjects. It seems to be a largest set of FISH painting data with exposure to chemical carcinogens (150 subjects) to date. We may postulate, under our conditions of a moderate exposure to chemical carcinogens and irradiation at NPP, we did observe specific differences between chemical exposure and irradiation in the pattern of chromosomal aberrations by FISH. The increase in reciprocal translocations as compared to controls seems to be specific for ACN and EB exposures, and in insertions for ACN, EB, and c-PAHs exposures. It seems to be more difficult to speculate about the specificity of chromosomal aberrations induced on chromosomes #1 and #4. It appears that chromosome #4 may be more susceptible to the clastogenic activity of chemical carcinogens than chromosome #1. But this suspicion should be further explored.

We may hypothesize that FISH analysis using whole chromosomes painting may determine a specific pattern of stable chromosomal aberrations induced by chemical carcinogens. The ability to detect specific chromosomal aberrations in specific chromosomes

using FISH method may be a powerful tool for monitoring individuals at increased risk of cancer. In future we should try to standardize the FISH analysis using whole chromosomes painting and propose the interpretation of results for the human risk assessment.

5. Conclusion

Suitability of FISH painting as biomarker of early effect in the field of molecular epidemiology was proved.

All our results suggest that the FISH technique is more sensitive than the conventional cytogenetic analysis. It was particularly apparent in case of police officers exposed to the levels of air pollution common in urban environment. In contrast to the FISH, the conventional method was not able to detect a chromosomal damage under these conditions.

The plausibility of this idea will be further examined in the future studies. However, we may put forward an idea that for the risk assessment for human health translocations may be evaluated as more significant chromosomal aberrations than breaks.

The connection between chromosomal aberrations measured by FISH and such biomarker of exposure as DNA adducts was established for the first time.

In addition, it could be hypothesized that FISH analysis using the whole chromosome painting may determine a specific pattern of stable chromosomal aberrations induced by chemical carcinogens. The ability to detect specific chromosomal alterations in specific chromosomes may be a powerful tool for monitoring individuals at the increase risk of cancer.

This study was utilizing a unique pool of human biological material.

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7. Abbreviations

ACN – acrylonitrile
B[a]P - benzo[a]pyrene
BER – base excision repair
CA – chromosomal aberrations
CGH – comparative genomic hybridization
CI – confidence interval
CYP1A1 - cytochrome P450, subfamily I (aromatic compound-inducible), polypeptide 1
CYP2D6 – cytochrome P450, subfamily IID (debrisoquine), polypeptide 6
CYP2E1 – cytochrome P450, subfamily IIE (ethanol-inducible), polypeptide 1
EB – ethyl benzene
EPHX1 – human microsomal epoxide hydrolase
$F_G/100$ – genomic frequency of translocations per 100 cells
FISH – fluorescence *in situ* hybridization
GSTM1 - glutathione S-transferase Mu 1
GSTP1 - glutathione S-transferase P1
GSTT1 - glutathione S-transferase theta 1
hOGG1 – human 8-oxo-guanine DNA glycosylase I
HPRT – hypoxantine-guanine phosphoribosyltrasferase
IARC – International Agency for Research on Cancer
ICRP - International Commission on Radiation Protection
IPCS – International Programme on Chemical Safety
mEH – microsomal epoxide hydrolase
mFISH – multicolour fluorescence *in situ* hybridization
MN – micronuclei
MTHFR – methylenetetrahydrofolate reductase
MS – methionine synthase
NAT1 and NAT2 – N-acetyltrasferases
NER – nucleotide excision repair
NPP – nuclear power plants
PAHs – polycyclic aromatic hydrocarbons
c-PAHs –carcinogenic polycyclic aromatic hydrocarbons
PBL – peripheral blood lymphocytes
PM2.5 - particulate matter that is 2.5 micrometers or smaller in size
RR – relative risk
RT-PRC – real time polymerase chain reaction
SKY – spectral karyotyping
XPD – xeroderma pigmentosum group D
XRCC1 – X-ray cross-complementing gene 1