

Cytogenetic biological dosimetry past, present and future perspectives

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Abstract. Human risk assessments at the low doses, and low dose rates and high doses following acute exposure to ionizing radiation are of prime importance in radiation protection. These issues are of continuing importance in respect of social/economic policy relating to the industrial and medical uses of ionizing radiation, and for risk assessment among people occupationally are being exposed to low and/or high LET radiation, such as astronauts, pilots, stewardess and nuclear power plant workers, as well as victims of radiation accidents. Consequently, several biological assays were developed and attempts were made to investigate formation of radiation induced chromosome aberrations and induction of genomic instability in human lymphocytes and fibroblasts. Fluorescence *in situ* hybridization (FISH) technique using chromosome, chromosome-arm, chromosome region, centromere and telomere specific DNA libraries has improved the resolution of detecting all classes of radiation induced chromosomal inter- and intra-changes. Consequently, has increased significantly the accuracy and detection limit of biological dosimetry. Newly obtained data indicate that (a) Premature chromosome condensation assay is a unique method to be used for immediate dose assessment at low (5cGy) as well as high doses (≥ 3 Gy) and can accurately discriminate between whole- and partial-body exposure in case of mass casualties and accidental over-exposure to high doses of ionizing radiation (b) FISH-based translocation assay has the potential to assess acute as well as chronic exposure in cases of accidental as well as occupational exposure to ionizing radiation, either immediately following exposure, or retrospectively by defining accumulative effects to red bone marrows. (c) there are distinct finger-prints (such as insertions and complex translocations) for high LET radiation in comparison to low LET radiation. The importance of these findings and future perspectives for biological dosimetry are discussed.

KEYWORDS: Cytogenetic assays, Ionizing radiations, Occupational and accidental over-exposure, Chromosomal alterations, Genomic instability, Biological dosimetry

1. Introduction:

Cytogenetics and radiation biology share a long history of more than seven decades. Virtually every radiological principle was either discovered or substantially verified through cytogenetic study. Numerical and structural chromosome aberrations are directly associated with a number of important radiogenic effects. Moreover, it has been found that chromosomal instability is the hallmark of cancer [1, 2] even can be used as the precursor of cancer [3-5].

The chromosomal aberration is a very sensitive biological end-point, and reflects the effect of radiation-induced DNA damages on the whole genome. Chromosome alterations such as dicentrics, micronuclei in binucleated cells, premature chromosome condensation (PCC) in peripheral blood lymphocytes are being used to estimate the absorbed dose immediately following a radiation accident [6]. The limit of detection of these tests (for whole body exposure) was found to be approximately 20, 30 and 5 cGy, respectively. The PCC technique proved to be the most sensitive assay for discriminating between whole and partial body irradiation [7].

The technique of Fluorescence *in situ* hybridization (FISH) using chromosome specific paint probes, a pan-centromeric and –telomeric probe for whole genome (Figure 1), has opened up the possibility of detecting stable chromosome aberrations in unexposed individuals, and made it possible to perform retrospectively biological dosimetry [6].

It is essential to estimate dose of exposure for several reasons. In case of high exposures (> 1 Gy acute), information on doses assists in the planning of therapy and in altering physicians to likely deterministic health consequences that could arise in the following weeks and years. High doses of ionizing radiation clearly produce deleterious consequences in human, including, but not exclusively, cancer induction [8]. At low-doses of radiation (< 1 Gy) the situation is much less clear. In mice following exposure to very low dose rate of gamma rays (total of 20 mGy at a dose rate of 0.05 mGy/day) no direct correlation was found for life shortening and increased neoplasia [9], however, in

Techa River cohorts an higher incidence of solid cancer has been reported [10]. In general, the risks of low dose radiation are of great importance in relation to issues as varied as screening tests for cancer, the existing and nuclear power plants, occupational radiation exposure, manned space exploration and radiological terrorism.

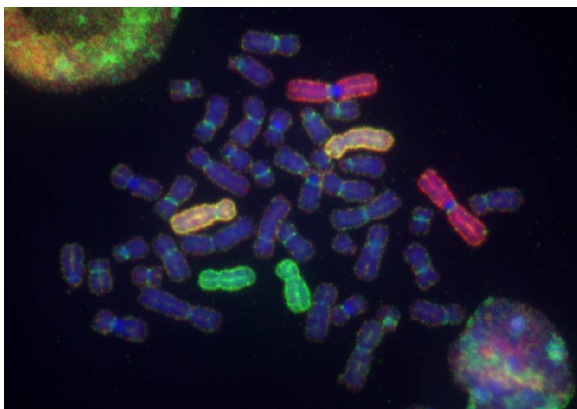
2. Methodology and rationale:

There are two major classes of chromosomal aberrations induced by ionizing radiation in cells at G₀ and G₁-stages of cell cycle. First, the classical unstable aberrations, which mainly include dicentric, acentric fragments and rings, and the second stable aberrations, such as translocations and insertions. By the mid 1960's it had been established that the frequency of dicentrics in peripheral blood lymphocytes can serve as an indicator of radiation exposure (a biological dosimeter)[6]. A large amount of data is available from in vitro studies using different radiation qualities, doses and dose rates, which could be used for calibration and thus for dose estimation [11-13]. In order to produce a dicentric chromosome aberration, DNA damage must be induced in the two unreplicated chromosomes involved as such that the damaged chromosome can undergo exchange. The exchange in part occurs as a result of mis-repair of the DNA damage, namely DNA double-strand breaks. X-rays and Gamma rays have very low LET (Linear Energy Transfer) with low frequencies of ionizations per unit track length. Thus, there is low probability that two forms of ionization from single track will occur within the target, and two ionization forms, at a minimum, are necessary to produce in the two chromosomes involved in a dicentric. The dose response curve for low LET radiation following acute- and high dose rate-exposure is generally assumed to fit the equation: $Y = \alpha D + \beta D^2$ where Y is the yield of dicentrics, D is the dose, α is the linear coefficient and β is the dose-squared coefficient. For low LET radiation following chronic- and low dose rate-exposure, as well as for high LET radiation (at both low- and high- dose rates) the conversion of chromosomal aberrations (i.e. dicentrics and translocations) yield to dose follows a simple linear equation: $Y = c + \alpha D$. Y is the yield of dicentrics or translocations, c is the control level, and α is the induction coefficient per unit dose and D is the dose.

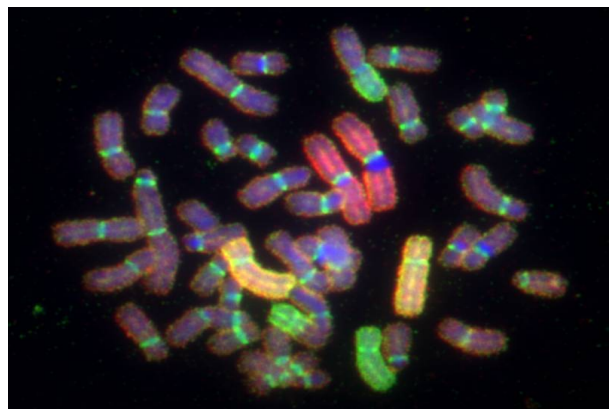
As the LET of radiation increases, there is a greater probability that two lesions within the target will be induced by two types of ionization along the same track resulting in a dicentric. Therefore, the dose response curve at higher LETs (above 20 keV/μm) will be linear. Dicentric analysis is being used for radiation dose assessment immediately following exposure. However, difficulties in dose estimation arise for the past exposure, due to a decline of cells containing unstable chromosome aberrations [6]. Fluorescence in situ hybridization technique employing chromosome specific DNA libraries to "paint" individual human chromosomes has opened new perspectives for rapid and precise detection of stable chromosome aberrations such as translocations in somatic [14, 15] and germ cells [16]. The inherent stability of translocations over cell generations has enabled them to be used as a biodosimeter [6, 17-19].

Figure 1: FISH-based translocation assay: Chromosome specific DNA libraries for chromosomes, # 1 (Biotin, red), # 4 (Biotin and FITC, yellow), #8 (FITC, green) + Centromeric probe for whole genome (FITC, green).

I. A normal metaphase.



II. A reciprocal translocation between chromosome #8 and one unpainted (DAPI, blue) chromosome.



These biological assays have been employed to study incidences of chromosomal alterations in control population (unexposed) and for dose assessment in several cases of radiation accidents.

3. Frequency of spontaneously occurring chromosomal alterations, and modulating factors:

In human peripheral blood lymphocytes, predominantly four cytogenetic biological assays, micronuclei (MN) in binucleated cells; premature chromosome condensations (PCCs) in interphase cells; dicentrics and translocations in metaphases are being used for dose assessment following exposure to ionizing radiation. Among these assays the control level (unexposed samples) of chromosomal alterations is in order of: micronuclei (3-35) > translocations (0-15) > dicentrics (1-4) > PCC (0-2) per 1000 cells analyzed [6].

For both MN and translocation assays influence of age on increasing level of chromosomal alterations was evident [6, 20, 21]. Moreover, smoking status suggests an acceleration of translocation \geq age of 60 years old [21]. Consequently, in cases of immediate and retrospective dose assessment using FISH-based translocation assay, the background frequency (age of exposed individuals) should always be considered.

4. Post Chernobyl Experiences (in human studies):

Chernobyl accident (in April 1986) essentially complicated ecological situation in Russia, Belarus and Ukraine on the background of the existent environmental pollution and enhanced the enlargement of population contact with ionizing radiation. The immediate medical response involved containment of the radioactivity, evacuation of the nearby population and the next step consisted of assessment of radiation dose received by individuals. Consequently, series of cytogenetic studies were undertaken in peripheral blood lymphocytes in children and adults, to estimate the dose of exposures in employee of the Chernobyl nuclear power plant personnel, in patients recovered from acute radiation sickness (ARS) in clean up workers, in evacuees and in population living in contaminated areas (i.e. self-settlers from 30-km alienation zone).

These studies were initiated immediately and in some cases follow up studies and retrospective dosimetry were performed using FISH-based translocation analysis. The notion was to estimate the frequency and spectrum of chromosome aberrations (including specific cytogenetic markers of radiation exposure) and to help clinician for therapy regimen of the victims.

In studies performed in the period of 1986-1987 in different groups dicentric analysis was used to estimate individual doses that ranged from 20 cGy to 980 cGy [22]. In the follow up studies in the same cohort or other groups (i.e. evacuees from Prypiat) higher incidence of chromosomal aberrations as detected by either by using standard G-banding or FISH-based translocation assay was evident in comparison to matched control group, though the frequency dicentric decreased and reached to the level found in unirradiated group [23-25].

Retrospective dosimetry in peripheral blood lymphocytes of individuals living in contaminated areas around Chernobyl and the Estonian clean-up workers seven years after accident was performed. The first study group composed of 45 individuals living in four areas (i.e. Rechitsa, Komsomolski, Choiniki, and Zaspas) in the vicinity (80-125 Km) of Chernobyl and 20 individuals living in Minsk (control group)(340 Km from Chernobyl). The second study group (Estonian cleanup workers) composed of 51 individuals involved in clean up the Chernobyl for a different period of time (up to 7 months) and a matched (i.e. age, sex, smoking and drinking habits) control group consisted of 14 probands. Unstable aberrations (dicentrics and rings) were scored in Giemsa stained preparations and stable aberrations (translocations) were analysed using DNA specific libraries and fluorescence in situ hybridization (FISH) technique.

On the basis of the dose response curve established (in vivo) for the victims of Goiania accident [17], the dose estimates presented constitute an estimate of the average exposure, and it was found to be in the range of 0.18 up to 0.4Gy [19, 25]. The calculation of the biological radiation doses on the frequency of reciprocal translocations using 3-4 chromosomes (representing approximately 20% of the genome) showed that FISH technique can be used for retrospective group dosimetry for the doses above 25 cGy (one should score 1000 cells, genome equivalent) and for the individual dosimetry 2000

cells genome equivalent is recommended. For situation of exposure in dose range $\sim 30 - 200$ cGy, 1000, and 500 cells genome equivalent, respectively should be sufficient, taking into account the factor of aging [18, 20, 21].

In order to reduce number of cells for analysis and to detect all structural as well as numerical aberrations simultaneously in one preparations a multi-colour FISH technique is developed and applied for whole genome analysis of human chromosomes using 23/24 different colours for 23/24 different human chromosomes [26].

Among the people living in the contaminated areas in the vicinity of Chernobyl, a higher frequency of numerical aberrations (i.e. trisomy / hyperdiploidy) was evident [19]. This is an important observation because generally all tumours were found to be aneuploid [3]. In a recent study using Mayak cohorts (ex-nuclear plant workers) occupational exposed to low doses of ionizing radiation also an higher incidence of numerical aberrations was evident.

5. Chernobyl experiences on genomic instability:

Introduction of modern FISH technique essentially improved the possibilities of cytogenetic monitoring in terms of the discovery of stable chromosome aberrations (including clones). Clonal del(22q) chromosome aberrations, a morphology similar to a Philadelphia chromosome were coincidentally observed in highly exposed (1.1 to 5.8 Gy) reactor personnel of the Chernobyl power plant accident in the course of retrospective biological dosimetry [27]. A rearrangement of the BCR gene on 22q11 could be confirmed in unstimulated peripheral blood by RFLP analysis from three of four del(22q) carrying cases. However, typical clinical leukemic symptoms associated with the translocation (9;22)(q24;q11) could not be found.

Jones and co-workers [28] reported on application of three somatic genetic biomarkers [translocations by FISH, hypoxanthine phosphoribosyltransferase (HPRT) mutant frequency by cloning and for glycophorin A (GPA) by flow cytometry] and covariates in radiation exposed Russian cleanup workers (625) of the Chernobyl nuclear reactor 6-13 years after exposure. The results revealed higher frequency of translocations and HPRT but not for GPA. Translocation analysis is found to be the preferred biomarker for low dose radiation dosimetry given its sensitivity, relatively few covariates, and dose response data. However, based on the estimated dose (9.5 cGy), the risk of exposure-related cancer is expected to be low.

In a similar type of study performed in other cohorts 13-15 years after accident, only translocation yields in exposed groups were found to be higher, leading to dose estimates of 0.2Gy. Micronuclei, apoptotic cells and HPRT analysis failed to reveal any difference with the matched control group [29]. Dubrova and co-workers [30] studied germline mutation at eight human minisatellite loci CEB1, CEB15, CEB25, CEB36, MS1, MS31, MS32 (loci D2S90, D1S172, D10S180, D10S473, D1S7, D7S21, and D1S8), and B6.7 (located on chromosome 20q13), chosen for their high spontaneous mutation rate, among families from rural areas of the Kiev and Zhytomir regions of Ukraine, which were heavily contaminated by radionuclides after the Chernobyl accident. A statistically significant 1.6-fold increase in mutation rate was found in the germline of exposed fathers, whereas the maternal germline mutation rate in the exposed families was not elevated. These data, together with the results of previous analysis of the exposed families from Belarus, suggest that the elevated minisatellite mutation rate can be attributed to post-Chernobyl radioactive exposure.

Results of other investigation [31], dealing with 7 hyper variable minisatellite loci CEB1 (D2S90), CEB15 (D1S172), CEB72 (D1S888), CEB42 (D8S358), CEB36 (D10S473), CEB25 (D10S180) and B6.7 revealed only a tendency to increased mutation rate without statistical significance. Authors drew a conclusion about mutagenic influence of irradiation only at the spermatogenesis cycle at the meiosis stage. To test whether ionizing radiation can cause paternal genetic mutations that are transmitted to offspring, 88 families of Chernobyl clean-up workers exposed to ionizing radiation were studied [32]. Mutation via DNA blotting with the multi-locus minisatellite probes 33.6 and 33.15 and via PCR in a panel of six tetranucleotide repeats were analyzed. Children conceived before and children conceived after their father's exposure showed no statistically significant differences in mutation frequencies. An increase in germline microsatellite mutations after radiation exposure was found that was not statistically significant. No dependence of mutation rate on increasing exposure was found. A novel finding was that the tetranucleotide marker D7S1482 demonstrated germline hypermutability. Data

received do not support an increased level of germline minisatellite mutations but suggest a modest increase in germline mutations in tetranucleotide repeats. Statistical power, however, was limited by small sample size.

Very high mutation rate of DNA was found in the offspring of Chernobyl accident clean-up workers with absorbed doses of 10-70 cGy (average 39.44 cGy) [33].

6. Comparison to non-Chernobyl studies:

The Goiania radiation accident:

A good cohort for such a study is the victims of the radiation accident in Goiania (Brazil) [17, 34, 35]. In this accident which occurred in September 1987, a cesium-137 (^{137}Cs) therapy unit was broken, and the radioactive ^{137}Cs was distributed, as a result of which 249 individuals were contaminated either internally or externally. Immediately after detection of the accident more than 110 blood samples from affected victims were analysed for the frequency of dicentrics and rings in the lymphocytes. Since no dose response curve for a low dose rate of ^{137}Cs was available, a calibration curve generated for ^{60}Co gamma rays at a dose rate of 0.12 Gy/minute was used for dose estimation. Among 110 individuals analysed, 29 had an estimated dose of 0.4 and above (maximum estimated to be 5.3 Gy). Though most of the individuals received a non-homogeneous dose suggested by the presence of localised burns in the skin, all cases except six showed a Poisson distribution for aberrations. In this accident the exposure was complicated (fractionated, protracted and internal) and resulted in most cases in a distribution that was not distinguishable from Poisson.

The victims of Goiania radiation accident were analyzed immediately and followed for 10 years [17, 35]. The frequencies of dicentrics were determined immediately following exposure in 1987 and onwards, and translocations were determined since 1992. The frequencies of translocations were determined at different periods (from 1713 days up to 10 years) after accident, and compared with the frequencies of dicentrics determined immediately after the accident in the peripheral blood lymphocytes. Assuming equal frequencies of translocations and dicentrics, one can analyse the present data for the validity of such a relationship between dicentrics observed in 1987 and the translocations observed in 1993. An overall test, pooling over all individuals, the hypothesis "equal frequencies of dicentrics and translocations" can be rejected at a very high significance level ($p < 0.001$) for doses above 1 Gy. In contrast, when only doses < 1 Gy are considered dose estimates made using translocations (6 years after accident) were exactly overlapped with the estimates made immediately following accident using dicentric analysis. A similar conclusion is made in the follow up of Chernobyl cohorts [22].

Concerning the persistence of translocations in experiments performed during 1992 until 1997, no significant difference could be detected [17, 3], that indicates persistence of translocations. In general the average genomic frequency of translocations did not alter significantly between the years investigated. The frequency of hyperploidy cells tends to increase with time. A similar observation was made among populations living in the vicinity of Chernobyl [19].

Turkish radiation accident:

This accident occurred in December 1998 and 10 people were exposed to ^{60}Co -radiotherapy source. Five were heavily exposed to doses of 2 to 3 Gy and the other five were exposed to doses of 0.5 to 1 Gy. Samples of blood were available at 1, 4, 6, 9, 13 and 25 months after the accident for all ten persons. At 21 months samples are available for only eight of the individuals, of which four are in the heavily exposed group [36].

Translocation, dicentric and micronuclei (MN) in binucleated lymphocytes yields measured at different time points after the accident. Dicentrics and MN have significantly decreased with post-exposure time. Generally dicentric frequency reduced by 50% at four months post-exposure time and for MN drastic reduction was even more pronounced and reached to approximately 60%. The half-life of lymphocytes among this cohort was estimated to be 120 days.

The combinatorial labelling and ratio labelling, so called combined binary ratio labelling (COBRA) – FISH assay was applied for whole genome analysis in this cohort. Translocations at low dose range did not decrease significantly with time and at higher dose range declined slightly (20%-30%) at six months after exposure and remained constant until 25 months.

COBRA M-FISH observed translocations were predominantly complete and at the latest post-exposure time all were complete. Using either COBRA M-FISH and triple colour FISH, it was found that translocations are persistent if measured in stable cells only (i.e. having no unstable aberrations, such as dicentric) [36].

7. Insights into the sites of X ray and neutron induced chromosomal aberrations in human lymphocytes using COBRA-M-FISH.

COBRA multi-colour fluorescence in situ hybridization (MFISH) assay has been applied to study in vitro low (X-rays) and high LET (neutrons 1 MeV) radiation induced chromosomal aberrations in human lymphocytes. With X rays the dose-effect relationships for both dicentrics and translocations were linear-quadratic, whereas with neutrons these were linear. Among aberrant cells, average estimates of the minimum number of breaks was higher for neutrons than for X rays. Moreover, the induced chromosomal exchange patterns were more complex following neutron irradiation in comparison with X rays. COBRA-MFISH was found to have a greater resolving power over partial labelling for the accurate detection of complex translocations (more than two breaks occurred in two or more chromosomes) and insertions (two breaks generated in two different chromosomes). With neutrons the frequencies of both were higher than those induced by X rays, and for insertions their relative proportions to the total frequencies of translocations were independent of dose. These data suggest insertions and complex type translocations can be used as the 'signature' of high LET radiation [26, 36].

8. Conclusions:

In cases of acute exposure to ionising radiation when the exposed individuals can be reached within a short period of time, dicentric assay can still be employed for dose assessment. However, in cases of mass casualties and when people exposed to rather high doses (≥ 2 Gy) PCC is the method of choice. In cases of retrospective biological dosimetry and for dose assessment of cohorts occupational exposed to low doses of ionizing radiation, studies performed thus far for validating the FISH translocation assessment revealed that it can develop into a reliable system for retrospective biological dosimetry at low doses (in the range of 25 cGy). The lower limits for other radiation qualities (high LET, such as neutrons and heavy ions) are lower when measured in Gy. The measurement of translocations in human lymphocytes is capable of providing an estimate of the lifetime dose to an individual. Strictly, it is the average bone marrow dose that is measured. By far is the most sensitive biological assay to elucidate the effect of ionising radiation on induction of chromosomal aberrations immediately and retrospectively following exposure. The observation that translocation yield in stable cells is independent of the time of sampling implies that direct calibration should use stable cells only. On the few occasions that dose has been estimated by this technique, reasonable agreement has been achieved with the known circumstances of the irradiation.

However, the existing data on either using biological assays to define dose estimates and/or to classify exposed populations by assessing the observed biological end-points such as stable chromosomal aberrations and mini-satellite could not conclusively illustrate any relation between ionizing radiation induced DNA damages at low dose level and an increase in frequency of specific type of cancer. However, in these processes there are number of state of art assays developed in which can permit to get a definitive picture on these events in future in the follow up studies. Therefore, it is recommended to perform a battery of test system from biological assays to epidemiological studies in the same cohorts to correlate relationship between ionizing radiation over-exposure and onset of cancer in human.

9. Recommendation for future actions:

Concerning biological dosimetry the following criteria are importance in order to generate a solid set of data in case of mass casualties following a radiation accident, as follows: 1. To

unify technical protocols and scoring criteria [6], 2. To apply multi-colour FISH for whole genome analysis, furthermore, state of art technology such as multi-colour FISH (whole genome labeling, arm- and band-specific probe) will facilitate to detect different types of stable-type aberrations [37], 3. To develop systems for automatic scoring of chromosomal exchanges, dicentrics and translocations [38].

It is obvious that there is a clear relation between induction of chromosomal abnormalities and dose of exposure following treatment with ionizing radiation. However, in order to correlate the induction of chromosomal abnormalities and enhancement of genomic instability that may lead to cancer, there is a clear lack of information between initial set of experiments and follow up studies. Moreover, the biological dosimetry, mortality rate, and the frequency of cancer and noncancer diseases were not measured in the same population, instead different population was employed as cohorts. Therefore, it is recommended to joint efforts between studies in biological dosimetry and epidemiology in order to elucidate on the role of induced chromosomal aberrations, cancer formation and cancer site-specific risks in cases of radiation over-exposure [39-41].

In addition, perspective area of further investigations in the field of biological consequences of Chernobyl accident for human health as well as Mayak and Techa River cohorts (low dose chronic exposure) seems to be further work on the radiation induced chromosome instability (transmissible, hidden, delayed) not only in irradiated persons but in their progeny [42] and so called “bystander effect”, as well as the evaluation of possible connection between the genome structure damages both stochastic (oncopathology) and non-stochastic (multifactorial pathology) radiation effects.

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