Twenty-two years later: consistent dose estimation of an accidental overexposure by retrospective biological dosimetry

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TITLE PAGE

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Running title: Consistent biodosimetry after 22 years

ABSTRACT PAGE

Twenty-two years later: Consistent dose estimation of an accidental overexposure by retrospective biological dosimetry

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The goal of this study was to retrospectively estimate the exposure dose of a victim from the Lilo radiological accident in Georgia twenty-two years afterwards and to compare it to the original cytogenetics-based analysis performed in our laboratory. Similar types of studies have been published, notably involving victims of the Chernobyl, Goiânia and Tammiku accidents [(1–3)]. Nevertheless, their estimations were done after shorter periods of time post-exposure and in some cases, the exposure might not have been exclusively of an external nature [(1,2)].

In this study, Fluorescence In Situ Hybridization (FISH) was used to score chromosomal translocations in lymphocytes from a recent blood sample of the victim and the dose assessment was performed using our laboratory's FISH calibration curve. The resulting whole-body exposure dose of 0.35 Gy [0.12 ; 0.72] was similar to the one obtained when reanalyzing the original scoring data from 1997 (0.38 Gy [0.13 – 0.75]), suggesting that our current calibration curve could be used for relative dose estimations long time after external exposure.

INTRODUCTION

Retrospective biological dosimetry based on translocations in peripheral blood lymphocytes is the most accurate method to date that allows to reconstruct a biological whole-body dose from an exposure scenario occurring decades in advance. This is due to the stability of translocations over time, in stark contrast to other biodosimetry techniques such as the Dicentric Chromosome aberration Assay (DCA), based on dicentric frequency, which decays with time and is understood to be reliable only up to several months after exposure[4–7]. This occurs because dicentrics, which harbor two centromeres, can disrupt proper chromosomal segregation, rendering cells unstable and triggering cell death. Although DCA is the most sensitive technique, translocation analyses are usually employed for samples collected several months to many decades after exposure [8–13].

Nevertheless, translocation-based dose estimation is complexified by the fact that chromosomal translocations accumulate throughout life, their rate thereby increasing with age. Moreover, there exist additional confounding factors that could affect the rate of translocations in individuals, such as their tobacco-smoking habits [7,14–17].

The circumstances of the Lilo accident have been previously reported [18,19]. In summary, after the dissolution of the Soviet Union, a military base in Lilo, Georgia was transferred to the Georgian government in 1992 and became a training center for Georgian frontier troops. The center had been used in the past for civil defense training by Soviet forces, where different types of radiation sources may have been used for equipment calibration and for training in radiological survey in the event of a nuclear attack or nuclear war [19]. Many radioactive sources, including twelve ^{137}Cs, one ^{60}Co and two hundred ^{226}Ra sources, many of which presented high dose rates, were abandoned by the military owners of the site without providing a thorough inventory and thus, were unaccounted for.

In 1997, local radiation-related skin lesions appeared on different parts of the bodies of eleven frontier guards and the IAEA was called upon to evaluate the radiological situation by the national government. The victims developed further lesions and severe symptoms, and some of them were transferred to specialized hospitals in Germany, Russia and France for treatment. Some of them had to undergo extensive plastic surgery and according to the IAEA report, because of the nature of the irradiation, internal organs, the bone marrow and the reproductive system were undoubtedly exposed [19]. Biological dosimetry analyses were performed in 1997 in order to assess the mean whole-body dose received by the 11 victims. At the time, a range of whole-body exposure doses from 0.1 to 1.3 Gy was estimated by DCA on peripheral blood lymphocytes. However, it is necessary to keep in mind that these values are not comparable to a case of localized irradiation. For example, a 32.5 Gy exposure to the skin was calculated by Monte Carlo dose simulation for one of the accident victims [19]. Furthermore, since the irradiation is supposed to have occurred over a long period of time (several to many months, depending on the victim), one should not underestimate the significance of the "whole-body" values, which are already high for some of the victims. A translocation-based analysis was also done for a fraction of the victims in our laboratory and not-surprisingly the results were comparable to those obtained with DCA [18]. In fact, for one of the victims, who was intermittently in close contact to a ^{137}Cs source (164 GBq, dose rate: 13 mGy/h measured at 1 m[19]) for an approximate period of 5 months, both the DCA and the FISH analyses performed in late 1997 resulted in a dose assessment of 0.7 Gy [0.5 ; 0.9] using the calibration curves of the laboratory at the time.

As part of a medical follow-up of this victim from the Lilo accident, a sample was recently received in our laboratory and a cytogenetic analysis was thus performed 22 years after the original exposure. Since the initial translocation and dicentric frequencies and their associated doses from the original sample analysis are traceable in our laboratory's archives, a direct comparison using our current methodology

and personnel was possible. In addition, this allowed to clarify an inconsistency observed in a previous publication from our lab[18].

Fortunately, real-life situations of radiological exposure are rare. And situations where dose assessments can be done through time on the same individual are even rarer. It is why sharing this type of case-report with the scientific community is of great importance to us.

MATERIALS AND METHODS

Cell culture

A peripheral blood sample of 7.5 mL was collected following informed consent from the patient (male, age 42, former smoker, previous medical exposures accounting for a total effective dose of ~22 - 41 mSv using reference values from the ICRP[20]) and cultures were set up in accordance with international recommendations[12] as previously described [7,18,21,22] with the following modifications. In short, 5 mL of PB-MAX™ Karyotyping Medium (Thermo Fisher Scientific, Courtaboeuf, France) were added to 0.5 mL of whole blood less than 48 hours after collection. In order to exclusively analyze first division cells, bromodeoxyuridine (BrdU; Sigma-Aldrich, St Quentin Fallavier, France) was added to the culture medium to a concentration of 0.05 mg/mL. Cells were then incubated for 48 h or 50 h at 37 °C. 2 hours before harvest, 50 µL of 10 µg/mL demecolcine (KaryoMAX™ Colcemid™; Thermo Fisher Scientific, Courtaboeuf, France) were added to the cultures. Harvest and fixation of cells were performed as previously described[21]. Finally, cell metaphases were spread onto clean glass slides.

Fluorescence *In Situ* Hybridization (FISH)

The FISH technique was performed as previously described[7]. A customized whole chromosome probe mix, containing probes for chromosomes 2 (orange), 4 (green), and 12 (orange and green) was used (XCP-Mix D-1100-202-MC; MetaSystems, Altlussheim, Germany). 10 µL of the XCP-Mix probe solution were applied to each slide, covered with a coverslip and sealed with Fixogum Rubber Cement (Marabu, Bissingen, Germany). Nuclear DNA was denatured by incubating the slides at 75 °C for 2 min, followed by an overnight incubation at 37 °C in a NeoBrite system (CliniSciences, Nanterre, France). Slides were washed once with 0.4x saline sodium citrate solution (SSC; VWR International, Fontenay-sous-Bois, France) for 2 min at 72 °C and once with 2x SSC supplemented with 0.05 % Tween® 20 (VWR International, Fontenay-sous-Bois, France) for 30 s at room temperature. VECTASHIELD® Antifade

Mounting Medium with DAPI (Vector Labs, Burlingame, CA, USA) was used for mounting and counterstaining.

Scoring criteria

Only stable metaphases (with no discernible dicentrics or fragments involving the painted chromosomes) exhibiting the three painted pairs of chromosomes (six colored centromeres) were considered for analysis. A bicolor chromosome exhibiting a single painted centromere accompanied by a bicolor chromosome exhibiting a single counterstained centromere was classified as a complete two-way translocation [7,21]. If only one bicolor chromosome was observed, the translocation was classified as incomplete (or one-way). Total translocations included all, complete and incomplete translocations. Expansion of translocation frequency to the whole genome was calculated using the following formula, as previously described [23,24]:

$$\frac{Fp}{FG} = 2.05 \sum_{i} fi\,(1 - fi) - \sum_{i<j} (fi * fj)$$

where *Fp* is the translocation frequency measured by FISH, *FG* is the translocation frequency equivalent to the whole genome and *fi* is the genome fraction painted with each color *i*.

Dose assessment

X-ray calibration curve for FISH analysis

An international population background database published by Sigurdson and colleagues[15], which contains the translocation information of subjects unexposed to ionizing radiation other than radiation from natural background sources or routine personal medical diagnostic procedures was taken into account to create the calibration curve. The calibration curve also included data produced in our laboratory of non-irradiated and irradiated blood from anonymous, consenting volunteers (3 female: age

39, former smoker; age 34, non-smoker; age 19, smoking status unknown; and 1 male, age 46, former smoker) and following the general guidelines described by the IAEA[12]. *In vitro* irradiations were performed at 37 °C (water bath) in an in-house LINAC facility (Elekta Synergy®) delivering X-rays with a maximal energy of 4 MV at a dose rate of 0.5 $Gy.min^{-1}$, with a 30 x 30 cm irradiation field at 107 cm from the source. Calibration in dose to water was performed using a cylindrical 0.125 cc ionization chamber (n.4920) to confirm the radiation field mapping and associated dosimetry.

DCA calibration curve

To generate the DCA calibration curve, blood samples were exposed to a ^{60}Co source (ICO 4000, Fontenay-aux Roses, France) with a nominal activity of 145817 GBq as of 22 February 2002 at a dose rate of 0.5 $Gy.min^{-1}$. The kerma rate was determined using a Physikalisch-Technische Werkstätten (PTW) ionization chamber irradiated in conditions as used for the samples. Uncertainty regarding the kerma rate was 4 %. Following IAEA and ISO recommendations[12,25], the calibration curve data encompassed a range of 16 different experimental doses between 0 and 5.7 Gy, with a total of 37715 metaphases and any associated aberrations (dicentrics and centric rings) being manually scored. The coefficients of the linear-quadratic adjustment and their corresponding uncertainties are as follows, with Y being the frequency of translocations and D the dose in Gy:

$Y = (0{,}0010 \pm 0{,}0003) + (0{,}0399 \pm 0{,}0061)\, D + (0{,}0485 \pm 0{,}0029)\, D^2$.

Statistics

Based on the data from Sigurdson et al[15], a quasi-Poisson regression model was fitted with the R glm function (R Package stats version 4.2.1[26]) in order to describe the basal translocation frequency as a function of the age of the donors. For a given age, a prediction of the translocation background value

and its associated 95% confidence interval were derived from the fitted model using the R add_pi function (R Package ciTools version 0.6.1[27]).

The calibration curve-fitting routine (for both DCA and FISH) was adapted from the one proposed by H. Braselmann in the cytogenetic dosimetry IAEA manual[12]. In short, the values for all the experimental data points of the curve were considered in order to determine the best fit coefficients based on maximum likelihood as described by Papworth and Merkle[28,29]. In this manner, the best fit values for the curve coefficients are found by maximizing the likelihood of the observations by the method of iteratively reweighted least squares when assuming a quasi-Poisson distribution.

RESULTS

Fluorescence *in situ* hybridization (FISH) is commonly performed in biological dosimetry in order to reconstruct a dose from the scoring of stable chromosomal aberrations (i.e., translocations). It is not the go-to technique largely because of the uncertainties associated with an increasing number of basal translocations with age in addition to its lower sensitivity compared to DCA. Nevertheless, it is a useful tool and one of the only that can be deployed to obtain a biological whole-body dose in cases of exposure dating from long time beforehand. For this type of analysis, a laboratory generally uses a calibration curve generated by irradiating blood *in vitro* at different doses, which usually encompasses values between 0 and 3 Gy (for low-LET radiation curves)[8,12].

The dose estimation made in 1997 was based on a FISH calibration curve generated with different methods and for which a traceability gap was recently found. Thus, FISH-based dose estimations made with it today cannot be entirely guaranteed. In contrast, since the raw data with the number and type of aberrations and scored metaphases from 1997 was traceable in our records, it was considered reasonable by us to re-assess the exposure dose using a more recent calibration curve.

Following the standards mentioned above, a calibration curve based on 4 MV X-rays was created in our laboratory. It encompasses approximately 3.7 million metaphases (1243628 cell equivalents) for the dose 0 Gy, coming mainly from the dataset of a previous study[15], as well as from data recently generated by our team. It also includes 10550 metaphases in total for samples irradiated in our laboratory, exposed to a range of doses of 0.1 to 5 Gy as detailed in Table 1. The coefficients of the linear-quadratic adjustment of the curve and their corresponding uncertainties are as follows, with Y being the frequency of translocations and D the dose in Gy: $Y = (0.0051 \pm 0.0001) + (0.0433 \pm 0.0161)\ D + (0.0670 \pm 0.0087)\ D^2$.

In 2019, one of the patients involved in the Lilo accident was hospitalized in France for a medical follow-up. During the treatment of his radiation-related injuries, a blood sample was collected with the goal to compare the current retrospective biodosimetry method in our laboratory to the one applied in 1997 after the radiological exposure. Notably, the recently created FISH dose-effect curve was used.

A typical FISH analysis was performed on the sample, where 3 pairs of chromosomes (2, 4 and 12) were painted and similar protocols and scoring criteria to those of the 1997 analysis were applied. A total of 16 translocations (reciprocal and non-reciprocal) were observed after analysis of 1639 "stable metaphases" (with no discernible dicentrics or fragments involving the painted chromosomes). This number of translocations is significantly different from the expected number of translocations for an individual of the same age (Table 2), based on the study performed by Sigurdson and colleagues [15] (see materials and methods), suggesting a radiological exposure, as expected.

The translocation frequency in the whole genome was calculated by using a conversion factor of 0.3385 based on the studies of Morton and Lucas[23,24], thus obtaining a mean estimated frequency of 0.0288 translocations per metaphase (95% confidence interval: 0.0165 – 0.0468). The corresponding value for the initial FISH analysis made for the patient in 1997 was of 0.0312 (95% confidence interval: 0.0178 – 0.0506), showing translocation signal stability over the years since the confidence intervals are similar and the minimal differences being probably due to Poisson uncertainties associated with the sampling of 1 to 2 thousand metaphases using the FISH-3 technique. In addition, as the patient does not belong to the age classes that show a higher accumulation of translocations according to the results of Sigurdson et al[15], a significant difference on the translocation signal was not expected.

When plotting the translocation frequency observed in the 2019 sample in the current dose-effect curve, a dose estimate of 0.35 Gy [0.12 ; 0.72] was obtained. This value is consistent with the one calculated using the frequency observed in 1997 and the current curve: 0.38 Gy [0.13 ; 0.75] (Table 3).

Since DCA is widely accepted as the gold standard in retrospective biological dosimetry, it was important to compare the FISH results to the observations made by DCA in 1997. A dose of 0.7 Gy [0.5 ; 0.9] was estimated at the time, however, the DCA calibration curve from our laboratory has also evolved since then, encompassing now more than 9-times the number of scored metaphases, a wider range of doses and more data points. Using the original raw data from 1997, the current DCA curve was employed, giving a mean whole-body dose estimate of 0.58 Gy [0.33 ; 0.87]. This value is comparable to that of the present FISH analysis, thus further validating our FISH-based assessment.

DISCUSSION

This study opens the option of using the new FISH-based curve in inter-laboratory comparisons, exercises, and particularly in an eventual long-time follow-up of individuals, since an analysis could be performed right after exposure during an accident for example, and then periodically (every 5-10 years) throughout the patient's life in order to better understand the development of chromosomal aberrations through time.

It is likely that the observed translocations in the recent blood sample come exclusively from precursor cells of the bone marrow that were exposed to ionizing radiation at the time of the events, as more than a decade has passed since exposure[12]. This is consistent with the description of the irradiation scenario and the injuries observed in the victim, suggesting that precursor reservoirs such as the iliac crests were greatly exposed during many months as mentioned in the IAEA report of the accident[19]. Thus, it could be hypothesized that the dose estimation based on the circulating lymphocytes from 2019 reflects the dose absorbed by the precursor cells of 1997. As the two assessments (1997 and 2019) are quite close, it could be concluded that the dose to the circulating lymphocytes at a given time could be a good indicator of the dose received by the precursor cells in a similar exposure scenario.

Based on the results presented here, it would be reasonable to claim that the dose-effect curve used is a reliable tool for retrospective dose estimation, although there is room for improvement of its robustness, which is one of the continuous goals in our laboratory. Enrichment of the curve data generated *in vitro* will continue in a systematic manner, following the same guidelines used until now. Nevertheless, one should be cautious as other factors may influence or have an additive effect on the rate of translocations, such as age and smoking[14,15]. Additionally, when possible, the medical history of the individual should be analyzed to unveil any relevant genotoxic exposures (radiological or chemical), as they can create translocations that persist over time[8].

As explained above, a significant increase in the translocation signal was not expected for the individual of this case-report since his age at the time of sampling for the two analyses (20 and 42 years old) is still considered relatively low and the expected translocations for the age classes is not too far apart from each other based on data from an international study of background translocation frequency[15]. Therefore, the stable signal for translocations observed in our case-study is not surprising. Nevertheless, this surely would not have been the case if the estimation had been made for an older individual as shown in Table 2, where the 16 observed translocations would not be considered significantly different than the translocation background for a person 54 years old and above.

Even though the recent version of the standard ISO 20046 suggests that age-adjustment should be done to ensure that the dose estimates being made are based on a curve appropriate to the age of the person, it is quite laborious and rare to have a calibration-curve including enough data points corresponding to each potential individual. This points to the necessity to continue to develop new methodologies of curve adjustment. Despite the fact that our laboratory's curve was able to predict a comparable dose to that the original assessment, one option being explored by us is to eventually expand the way the analysis is done by looking at the correlation not only with the age of the individual, but simultaneously with other life-factors such as tobacco exposure, which has been shown previously to affect the relationship between translocation rate and age[15]. Application of different mathematical models is underway with the expectations that a more "personalized" assessment could be done.

In conclusion, data from the FISH analysis of the recent sample of the Lilo victim shows an estimated frequency of translocations present in circulating lymphocytes that well exceeds the background level for the general population. Furthermore, the dose estimation performed for this work, 22 years after irradiation, is consistent and very close to that obtained from the sample collected right after exposure by means of DCA and FISH. This comparison suggests the validation of the current calibration curve,

which could be used in case of an analysis of external exposure occurring years and even decades beforehand. This opens a way to explore the age-adjustment question when performing dose assessments for individuals, especially for those in the higher age classes.

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CONFLICT OF INTEREST

The authors declare no conflict of interest regarding the content of this study.

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TABLES

Table 1. Experimental data for the FISH 4 MV X-ray calibration curve.

Dose (Gy)	Cell Equivalents	Translocations
0	1243628	6309
0,1	668	11
0,25	505	7
0,5	500	21
0,75	321	29
1	775	97
1,5	263	53
2	203	68
3	171	96
4	41	51
5	82	185

Table 2. Comparison of the predicted number of translocations at different ages. Prediction model based on the data of Sigurdson et al[15]. Ages shown include those of the first analysis (20), second analysis (42) and the significance threshold for 16 observed translocations (54).

Age	Predicted number of translocations (for 1639 metaphases)	Lower limit of 95% Confidence Interval	Upper limit of 95% Confidence Interval	Statistically significant for 16 observed translocations?
20	1.7	0	8	Yes
42	3.6	0	12	Yes
54	5.6	0	16	No

Table 3. Dose assessments using the FISH 4 MV X-ray calibration curve

Year	Mean whole-body dose	95% confidence interval
2019	0.35 Gy	0.12 – 0.72 Gy
1997	0.38 Gy	0.13 – 0.75 Gy