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Monitoring Translocations by M-FISH and Three-color FISH Painting Techniques: A Study of Two Radiotherapy Patients

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M-FISH/FISH-3 painting/Chromosomal aberrations/Radiotherapy/Biological dosimetry.

Purpose: To compare translocation rate using either M-FISH or FISH-3 in two patients treated for head and neck cancer, with a view to retrospective dosimetry.

Materials and methods: Translocation analysis was performed on peripheral blood lymphocyte cultures from blood samples taken at different times during the radiotherapy (0 Gy, 12 Gy and 50 Gy) and a few months after the end of the treatment (follow-up).

Results: Estimated translocation yield varied according to the FISH technique used. At 50 Gy and follow-up points, the translocation yields were higher with FISH-3 than with M-FISH. This difference can be attributed to three events. First, an increase in complex aberrations was observed for 50 Gy and follow-up points compared with 0 Gy and 12 Gy points. Second, at the end of treatment for patient A, involvement of chromosomes 2, 4, 12 in translocations was less than expected according to the Lucas formula. Third, a clone bearing a translocation involving a FISH-3 painted chromosome was detected.

Conclusions: More translocations were detected with M-FISH than with FISH-3, and so M-FISH is expected to improve the accuracy of chromosome aberration analyses in some situations.

INTRODUCTION

Biological dosimetry is routinely used to estimate the dose of ionizing radiation received after accidental overexposure or suspected exposure. Analysis of dicentric chromosomes in peripheral blood lymphocytes gives a good dose estimate when biological dosimetry is performed shortly after accidental exposure. However, the number of dicentrics decreases with time, as they are lost through cell divisions. Therefore, for retrospective dosimetry, so-called stable translocations are preferred.[1–4] They can be easily detected using fluorescence *in situ* hybridization (FISH), which is classically applied to three chromosome pairs in so-called FISH-3 painting.[5–9] To be used for retrospective dosimetry, translocations have to be relatively stable over time, but literature reports are in conflict over this point.[10, 11]

Scoring translocations with FISH-3 could lead to observation of differences in the total translocation yield when compared with M-FISH results.[12,13] This could come from three possible sources.

First, an empirical formula is used to extrapolate to the full genome from the yield of translocations observed with FISH-3.[3] This formula relies on the hypothesis that the probability of interaction of one chromosome with another is dependent on the DNA chromosome content. However, it has been shown that some chromosomes are more or less radiosensitive, which means they are more or less involved in translocations.[14–18] The reason could be variations in the relative size of chromosome territories or in the nature and number of active genes carried by the chromosomes considered.[19–24] Furthermore, chromosome radiosensitivity also seems to depend on the donor, which could in part explain the controversy.[14]

Second, some chromosomal aberrations are not well detected by FISH-3. This is so for complex aberrations (resulting from at least three breaks in at least two chromosomes), which can be misclassified as apparently simple.[13] The influence of misclassification of complex aberrations may be quite significant.[25]

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Third, and less often, the presence of clones in the sample could significantly change the FISH-3 dose estimation. Indeed, if two cells each contains a translocation involving the same painted chromosome, it is difficult to know if these two cells are clones or not as the counterpart chromosome is not identified. Therefore, such cells could not be removed from FISH-3 scoring and clonal translocations would be included in the translocation yield that would then be increased.

The use of full genome staining (M-FISH) could overcome these biases of FISH-3.[26,27] The behavior of all translocations can be studied without being disturbed by individual chromosome radiosensitivity or by a limited number of translocations, as all inter-chromosomal exchanges are visible. The comparison between the two techniques has already been performed[13] on *in vitro* irradiated blood samples, but we wanted to study the utility of M-FISH in the case of *in vivo* partial irradiation. Furthermore, as most accidental radiation exposures are heterogeneous, we chose to analyze translocations using M-FISH in lymphocytes from patients treated with radiotherapy for head and neck cancer.

MATERIALS AND METHODS

Patients

The study was performed on blood samples from two patients treated for a head and neck cancer by radiotherapy, who gave their informed consent. Neither patient was treated by chemotherapy.

Patient A: male, 68-year-old heavy smoker (50 pack-year). A total dose of 50 Gy (Cobalt 60) was delivered to the tumor through lateral opposed fields of 332 cm² at a dose rate of 0.5 Gy/min in 25 fractions of 2 Gy.

Patient B: female, 45-year-old heavy smoker (30 pack-year). A total dose of 50 Gy (Cobalt 60) was delivered to the tumor through lateral opposed fields of 334 cm² at a dose rate of 1 Gy/min in 25 fractions of 2 Gy.

Blood samples were taken before treatment (0 Gy), after 12 Gy exposure (12 Gy; 8 days after the beginning of treatment), immediately after completion of irradiation (50 Gy, 53 (patient A) or 32 (patient B) days after the beginning of treatment), and four months (patient B) or six months (patient A) later (follow-up).

Culture conditions and chromosomal preparations

Whole blood cultures were set up as follows: 500 µl of whole blood was added to 5 mL RPMI 1640 medium supplemented with 10% heat-inactived fetal calf serum, 1% penicillin-streptomycin (stock solution 10000 IU/mL), 1% Hepes buffer (stock solution 1 M), 1% L-glutamine (stock solution 200 mM), 1% sodium pyruvate (stock solution 100 mM, all previous products were supplied by Invitrogen, France), and 1% 5-bromodeoxyuridine (5-BrdU, stock solution 0.5 mg/mL, Sigma, USA).

The lymphocyte cultures were stimulated with 150 µL phytohemagglutinin (PHA, Invitrogen, France) and were incubated at 37°C for 46 h. Colcemid (KaryoMAX, Invitrogen, France) was added to a final concentration of 0.1 µg/mL and the cultures were continued for 2 h.

After harvesting, the cells were treated by hypotonic shock (0.075 M KCl, Sigma, USA) for 8 min at 37°C and were fixed three times in 10 mL methanol:acetic acid mixture (3:1, v/v).

Cells were dropped onto clean slides, air-dried and kept at –20°C until preparation of slides for analysis by FISH methods.

Multicolor FISH

M-FISH was performed on fresh slides that were incubated overnight at 37°C. We used the "24Xcytes" probe-kit (MetaSystems, Germany), which contains chromosome probes labeled with specific fluorochrome combinations.

Fluorescent images were captured with a Zeiss Axioplan epifluorescent microscope connected to the ISIS/mFISH imaging system (MetaSystems, Germany).

FISH-3 painting

As for M-FISH, FISH-3 was performed on freshly spread slides containing metaphases and incubated overnight at 37°C. Slides were pretreated with 0.05% pepsin (Roche, Switzerland) for 10 min at 37°C and subsequently washed in PBS (Invitrogen, France) for 5 min. They were incubated in a post-fixation solution, 1% formaldehyde (VWR, France) and 50 mM $MgCl_2$ (Sigma, U.S.A) in 1X PBS for 10 min at room temperature, and then washed in PBS for 5 min. After dehydration (70, 90, 100% ethanol for 2 min each), the slides were denatured for 2 min in 70% formamide (VWR, France) at 65°C, dehydrated (70, 90, 100% ethanol for 2 min each), and air-dried.

Commercial whole chromosome probes for chromosome 2 (mix FITC:Cy3, Cambio, UK), chromosome 4 (Cy3, Cambio, UK), and chromosome 12 (FITC, Cambio, UK) were denatured for 10 min at 65°C and then incubated for 30 min at 37°C. Slides were hybridized overnight at 37°C before washing in 50% formamide at 45°C for 5 min and three times in 4X SSC plus 0.05% Tween at 45°C for 4 min.

Chromosomes were counterstained using DAPI/Antifade solution (Qbiogene, USA).

Fluorescent images were captured with a Zeiss Axioplan epifluorescent microscope connected to the ISIS/M-FISH imaging system (MetaSystems, Germany).

Chromosome aberration scoring

At least 200 cells were scored per sample for M-FISH, and 1000 to 1500 for FISH-3. However, for several samples, we did not succeed in scoring enough metaphases as we ran out of sample.

Only metaphases containing the full set of 46 chromosomes

were scored. For M-FISH and FISH-3, only apparently stable cells were scored, as recommended in a translocation stability study.[10] Therefore, cells with dicentrics, rings or fragments were excluded from the scoring. Furthermore, cells with truncated chromosomes were also excluded from the scoring.

For both FISH techniques, we considered total translocations as the sum of apparently simple two-way and one-way translocations. We also added apparently stable complex exchanges resulting from at least three breaks in at least two chromosomes in stable cells. As there is no consensus on the way to include stable complex exchanges in the total translocation yield for retrospective dose assessment in biological dosimetry,[28] we decided to break down stable complex exchanges into simple translocations.

Metaphases suspected to be a clone were eliminated, to avoid clonal cell bias.

To compare FISH-3 and M-FISH effectively, the number of translocations observed by FISH-3 was extrapolated to the whole genome following Lucas' formula described below. Morton's chromosome DNA content data were used to calculate F_G.[29,30]

$$F_G = \frac{F_{2,4,12}}{2.05\left[f_2(1-f_2)+f_4(1-f_4)+f_{12}(1-f_{12})-f_2f_4-f_2f_{12}-f_4f_{12}-f_2f_4f_{12}\right]}$$

F_G is the genomic translocation frequency, $F_{2,4,12}$ is the frequency of translocation detected by FISH-3 and f_2, f_4, f_{12} correspond to the fraction of the genome of each painted

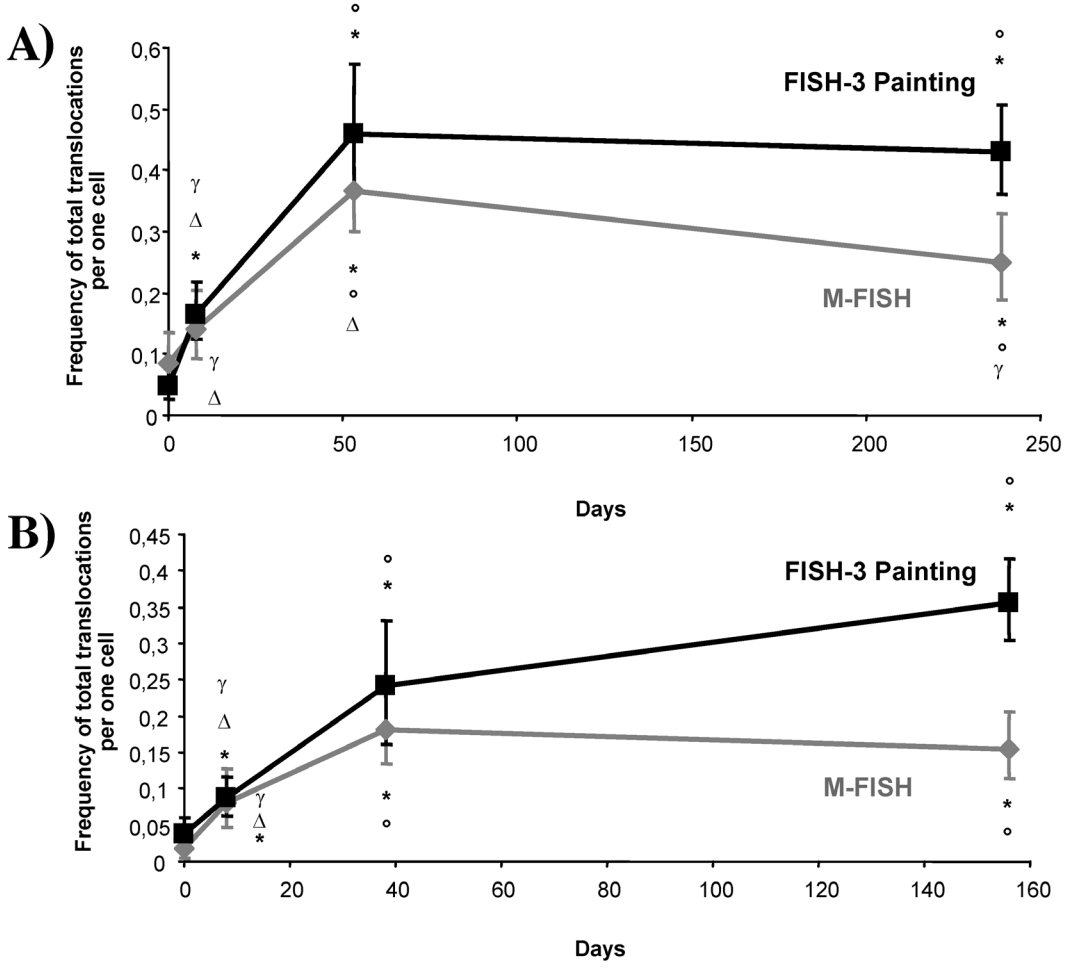

Fig. 1. Time-course of total translocation yield. The frequency of total translocations per cell was obtained by both FISH techniques before treatment (0 Gy; D0), after 12-Gy exposure (12 Gy; D8), after completion of radiotherapy (50 Gy; D53 patient A, D32 patient B), and several months post-irradiation (follow-up; D239 patient A, D156 patient B). Only stable cells were analyzed. Stable complex translocations were broken down into simple translocations in order to be scored. Error bars represent the 95% confidence interval according to Poisson statistics. Significance was tested using a specific chi square test adapted to Poisson statistics. *: statistically different from unirradiated ($p < 0.05$), °: statistically different from 12-Gy exposure ($p < 0.05$), γ: statistically different from end of treatment ($p < 0.05$), Δ: statistically different from 6 months after exposure ($p < 0.05$). Graph A for Patient A and B for Patient B.

chromosome.

Therefore, in our case the denominator is equal to 0.333 for a female genome and to 0.337 for a male genome. Thus, the whole genome yield can be estimated by multiplying by three the number of translocations detected by FISH-3.

Statistics

Confidence intervals, for each chromosome aberration yield, were calculated according to Poisson statistics. A specific chi square test adapted to Poisson statistics (described below) was used to estimate the statistical differences between groups of results.[31)]

This test calculates $\bar{y}$ for two translocation yields, y_1 and y_2. For each one, the number of translocations is x_1 and x_2 and the number of scored cells is t_1 and t_2.

$$y_i = \frac{x_i}{t_i} \; and \; \bar{y} = \frac{\sum x_i}{\sum t_i}$$

Then, for each translocation, yields y_i, c_i are calculated as described below:

$$c_i = 2(\sqrt{x_i + 1} - \sqrt{t_i \bar{y}}) \; if \; yi < \bar{y}$$

$$c_i = 2(\sqrt{x_i} - \sqrt{t_i \bar{y}}) \; if \; yi > \bar{y}$$

The statistical test $\sum C_i^2$ is approximately distributed as a χ^2 test with one degree of freedom. When the χ^2 limit is overcome then the two yields are significantly different.

Table 1. Stable complex translocations and clone translocation frequency per one cell for Patients A and B. (): frequency per one cell of total translocations. The total translocation is the sum of simple, multiple and complex translocations. Multiple translocations are simple translocations observed in the same cell. Complex translocations are translocations produced by at least three breaks in at least two chromosome. Insertions are complex translocations. Simple translocation frequency was calculated with aberrant cells containing only one simple translocation. Genome equivalent frequencies are FISH-3 translocations frequencies extrapolated to the whole genome by using Lucas's formula. Clonal translocation is a same translocation that is present in different lymphocytes.

	0 Gy		12 Gy		50 Gy		Follow-up	
	M-FISH	FISH-3	M-FISH	FISH-3	M-FISH	FISH-3	M-FISH	FISH-3
Patient A: Number of cells scored	210	998	193	954	263	503	207	951
Number of total translocations	18 (0.086)	16 (0.016)	27 (0.14)	58 (0.061)	98 (0.373)	83 (0.165)	59 (0.285)	142 (0.149)
Frequency of total translocations genome equivalent	0.086	0.048	0.14	0.183	0.373	0.5	0.285	0.448
Frequency of simple translocations genome equivalent	0.072	0.048	0.115	0.139	0.171	0.246	0.082	0.309
Frequency of stable complex and multiple translocations/total translocations	0.167	0	0.185	0.241	0.571	0.518	0.695	0.303
Frequency of stable complex and multiple translocations	0.014	0	0.026	0.015	0.213	0.085	0.198	0.045
Patient B: Number of cells scored	228	1536	223	1493	259	409	310	1396
Number of total translocations	4 (0.018)	20 (0.013)	18 (0.082)	43 (0.029)	47 (0.185)	33 (0.081)	48 (0.157)	166 (0.119)
Frequency of total translocations genome equivalent	0.018	0.039	0.082	0.086	0.185	0.242	0.157	0.357
Frequency of simple translocations genome equivalent	0.018	0.033	0.065	0.079	0.129	0.171	0.094	0.174
Frequency of stable complex and multiple translocations/total translocations	0	0	0.222	0.093	0.319	0.303	0.417	0.524
Frequency of stable complex and multiple translocations	0	0	0.018	0.003	0.059	0.024	0.066	0.062
Number of clone	2	/	4	/	5	/	5	/
Frequency of clonal translocations	0.009	/	0.018	/	0.019	/	0.016	/
Frequency of clonal translocations/total translocations	0.333	/	0.182	/	0.096	/	0.094	/

RESULTS

Chromosome aberration yields

Translocation yield varied according to the FISH technique used. With M-FISH, it increased during treatment of patient A and decreased at the follow-up point (Fig. 1A). The same trend was observed for the patient B, but the increase at 50 Gy was not as great, and the frequency of translocations at the follow-up point did not differ significantly from the frequency at 50 Gy (Fig. 1B).

By contrast, FISH-3 indicated significant increases in translocation yield during treatment of both patients (Fig. 1). At the follow-up point, the number of translocations was increasing in patient B, without being significantly different than at 50 Gy, whereas it remained stable for patient A.

Whereas the two patients were irradiated with the same protocol, the yields of radioinduced translocations obtained with both FISH techniques were differents especially concerning point 50 Gy and follow-up (Table 1).

Stable complex translocations

For both patients, the total translocation yield was higher with FISH-3 than with M-FISH for the points 50 Gy and follow-up, whereas no difference was observed for the points 0 Gy and 12 Gy (Fig. 1). Interestingly, for the points 50 Gy and follow-up, the stable complex translocations and multiple translocations (cells containing several independent translocations) represented between 30% and 60% of the total translocations (Table 1). Such increase in complex

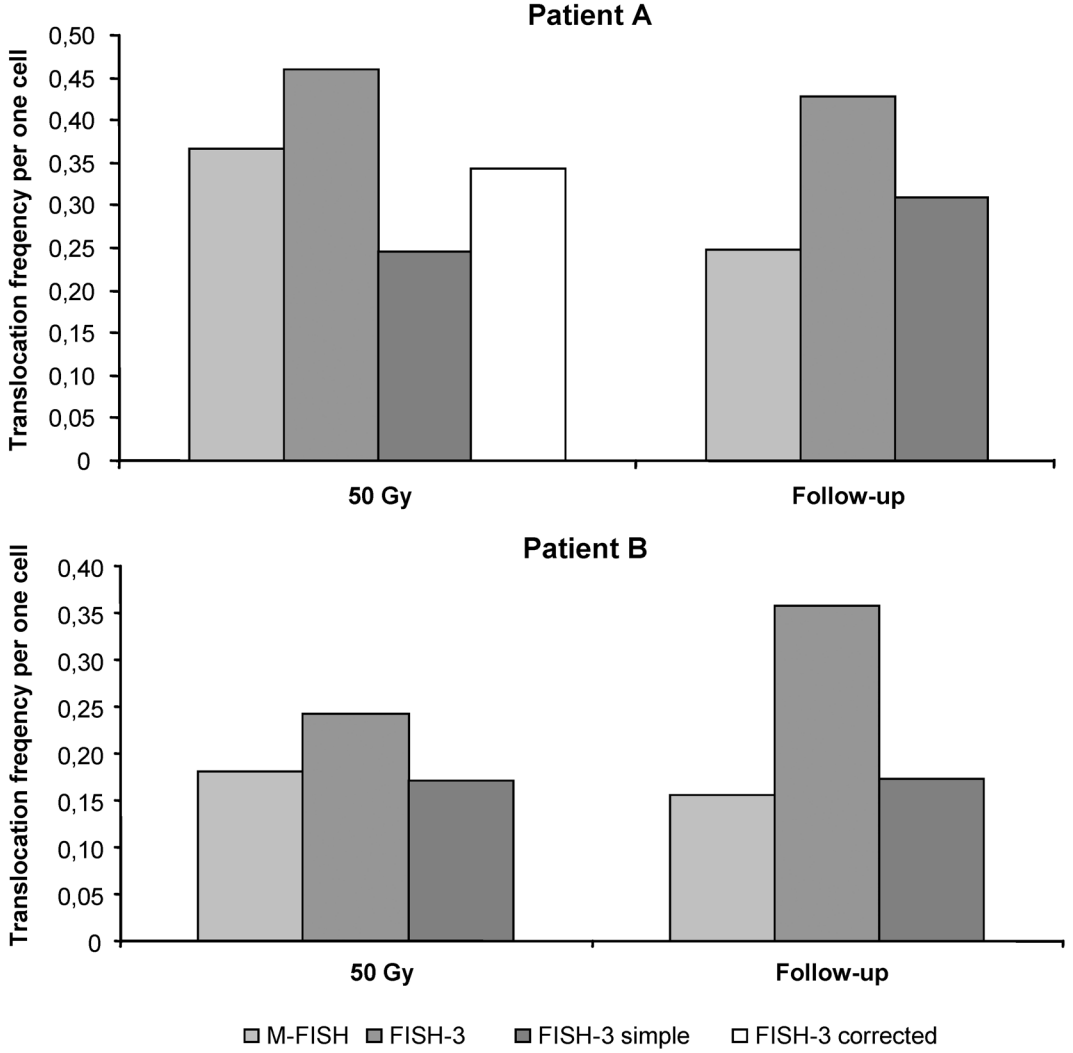

Fig. 2. Comparison of translocation yields. The frequency of translocations per cell was obtained by both FISH techniques after the end of the treatment and six months later. Only stable cells were scored. M-FISH and FISH-3 correspond to total translocation yield, simple FISH-3 to simple translocation yield, and FISH-3 corrected to the simple translocation yield where the extrapolation to the genome equivalent was obtained by multiplying by 1/0.24. Graph A for Patient A and B for Patient B.

aberration after radiotherapy was also observed by Durante *et al.*[32] Thus, the increase in the difference in total translocation yield between the FISH techniques seemed to be correlated with the increase in stable complex and mutiple translocations.

In order to confirm these findings, stable complex and mutiple translocations were removed from the FISH-3 scoring. No more differences were observed between FISH-3 and M-FISH for the two follow-up points of the two patients and for the 50 Gy point of patient B (Fig. 2). However, a difference remained for the 50 Gy point of patient A. This point will be discussed later. Therefore, stable complex and multiple translocations identified by FISH-3 could be one reason for the differences observed between the two FISH techniques.

Furthermore, when stable complex and multiple translocations were removed from M-FISH scoring, then the yield of simple translocations obtained were lower than FISH-3 translocation yields for points 50 Gy and follow-up (Table 1). Thus, the miclassification of stable complex and multiple translocations by FISH-3 as simple translocations was quite important.

Interestingly, the difference between patients simple translocations yield matches the difference of simple translocations background (Fig. 3). The main source of differences between both patients were the complex and multiple translocations yield.

Chromosomal involvement in translocations

To check if the difference observed between the techniques was related to chromosome radiosensitivity, the involvement of chromosomes 2, 4 and 12 was calculated from M-FISH results for each point. Interestingly, for the 50 Gy point of patient A, the calculated involvement was found to be 24% whereas the expected one was 33%. Even if this difference was not significant, if chromosomes 2, 4 and 12 were less involved than expected in this sample, this could explain why fewer simple translocations were observed for this point with FISH-3 than with M-FISH. The FISH-3 genome equivalent frequency of simple translocation was then calculated by multiplying the FISH-3 simple translocation yield by 1/0.24 instead of by 1/0.33 (Fig. 2A). Doing this, the genome equivalent simple translocation yield was the same than the M-FISH translocation yield. Therefore, a change in the involvement of chromosomes 2, 4, 12 could lead to an underestimation of translocation yield with FISH-3.

According to our results, a change in the probability of chromosome interaction could arise randomly, leading erroneous estimation of translocation yield by FISH-3. This is confirmed by reported inter-individual variability in chromosomal involvement when scoring translocations.[14] Fortunately, M-FISH is not sensitive to variations in expected chromosomal involvement in translocation.

Lymphocyte clone

A lymphocyte clone, with a two-way translocation involving chromosomes 2 and 5, was identified in patient B by M-FISH. This clone was present before radiotherapy and its ratio of 1.5% analyzed cells did not significantly vary during the study (Table 1). However, the bias in translocation yield induced by this clone was estimated using M-FISH. If the clones were included in the scoring, the translocations originally from clones represented about 33% of total translocations at the 0 Gy point and 9.4% at the follow-up point

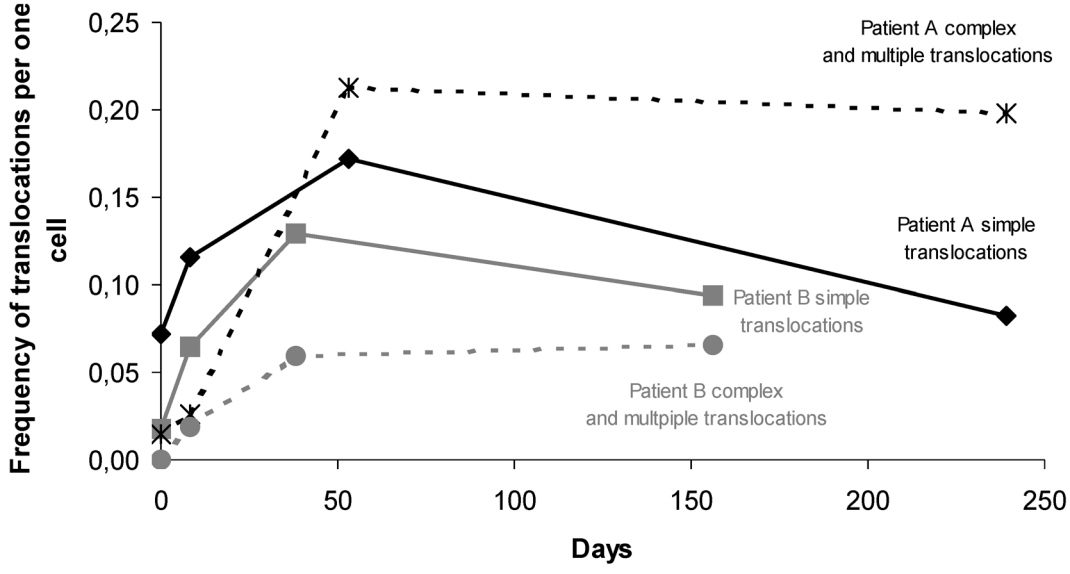

Fig. 3. Time course of simple translocations and of complex and multiple translocations. This frequencies per one cell were obtained by M-FISH.

(Table 1). When these findings were extrapolated, from the M-FISH results, to determine the impact of the clone on the FISH-3 results, translocations originally from clones represented about 60% of total translocations at the 0 Gy point and 24% at the follow-up point (data not shown). Therefore, if the translocations involved in the clone were added to the radiation-induced translocations, the whole genome translocation frequency evaluated by FISH-3 would have been even higher than that observed with M-FISH.

DISCUSSION

We have found that stable complex cells identified by FISH-3 seem to be a source of translocation yield estimation errors. With FISH-3, a stable complex aberration formed by at least three translocations should always be visualized, but either as a simple or as a complex translocation. When converted to the full genome, a stable complex aberration identified, for example, as three translocations with FISH-3, corresponds to nine genome equivalent translocations. Whereas according to our M-FISH observations, stable complex cells rarely contained more than four translocations. Obviously, there is a bias induced by converting stable complex translocations detected by FISH-3 to genome equivalent (Fig. 4). This bias should also concern multi-aberrant cells, and would lead to an overestimation of the total translocation yield by FISH-3, as has been suggested.[13)]

It seems interesting to remove stable complex and multiple translocations from FISH-3 scoring. But without such aberrations, it would not be possible to use mathematical models in the case of heterogeneous irradiation, as the distribution of chromosomal aberrations is needed.[33)]

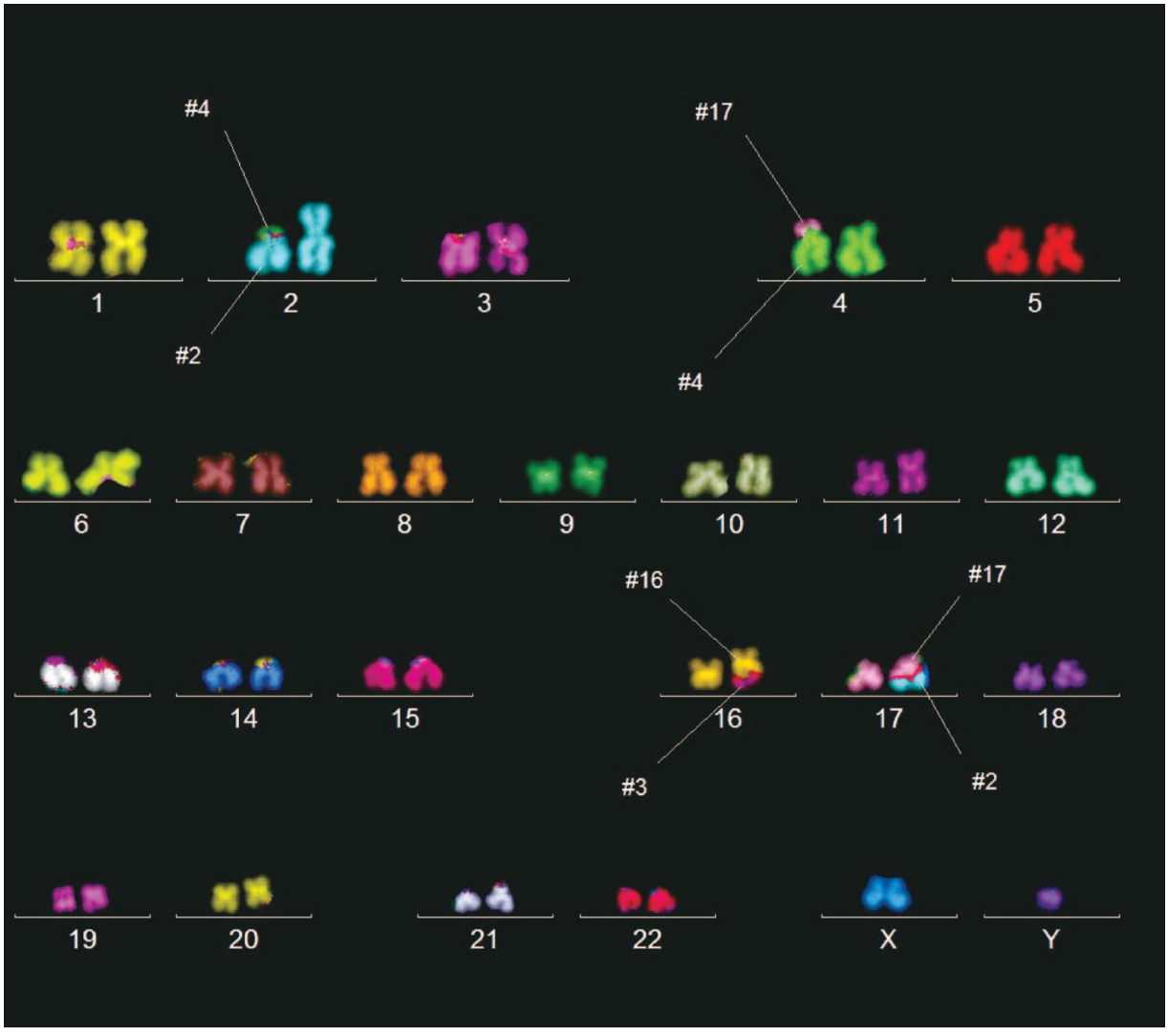

Fig. 4. Stable complex translocation visualized by M-FISH. This stable complex translocation is visualized as the sum of 4 simple translocations and could also be visualized by FISH-3 as the sum of 3 simple translocations.

In our study, whatever the FISH technique used, samples taken after irradiation contained complex or multi-aberrant cells, whereas samples taken before contained very few or no such aberrant cells (Table 1). Furthermore, in some studies it was shown that the background of complex aberration was equal to zero for unirradiated lymphocytes.[34–39] The same findings were obtained concerning multiple translocations.[40,41] Thus the presence of complex or multiple translocations seems to be used in order to distinguish irradiated samples from unirradiated ones. The complex translocation observed for patient A at the 0 Gy point with M-FISH could be explained by the two computed tomography scans using iodinated contrast product that were done before the treatment. In fact, iodine are photoactivated by computed tomography X-rays resulting in the increase of the ionizing radiation damages.

Furthermore, as the complexity of such aberrations is dose-dependent, such cells could be used to give information relative to the degree of blood exposure.

Additionally, it was shown that stable complex translocations are more specific to densely ionizing radiation than simple translocations.[36] In the same way, Anderson *et al* suggested that insertions could be used as a biomarker of high LET particles.[25] Thus complex translocation could give information on the quality of ionizing radiation.

In conclusion, if stable complex cells were added to the scoring, it would increase the dose estimation accuracy. According to this observation and our results, when stable complex translocations are numerous, M-FISH seems to be a better technique to study translocations than FISH-3.

The metaphase selection process used could contribute to the differences observed in translocation yield between the two FISH techniques. With M-FISH, the analyses were performed on karyotyped metaphases. Therefore, metaphases were rejected because of overlapping chromosomes that did not allow karyotyping, or because of missing chromosomes. Whereas with FISH-3, only the three painted chromosomes were analyzed in detail, and the quality of the other chromosomes was not so important. Therefore, fewer metaphases were rejected, as has been described elsewhere.[12]

Detection and rejection of unstable cells was better done with M-FISH than with FISH-3, as all chromosomes were carefully analyzed during karyotyping. This is particularly important in the case of follow-up studies, as unstable aberrations may impact on the behavior of translocations.[42] Thus, more metaphases with aberrations, as they were unstable, were excluded with M-FISH, while they were scored with FISH-3.[12]

Our observations suggest that if FISH-3 alone had been done on the patient B samples, the clone would not have been detected. The operator could not easily identify the presence of a clone with a translocation involving only one painted chromosome without any indication of the nature of this translocation.

Clones may represent up to 50% of peripheral lymphocytes,[43,44] but are in general rare, and involve only a few percent of circulating lymphocytes. However, the dose estimation error is still important as the translocations carried by clones can represent up to 50% of the total scored translocations.[45] Clones bearing translocations can be observed in exposed and nonexposed populations.[44–47] The emergence of clones seems to be mainly related to the age of the subject and to the dose received.[44,45,47]

Thus, the results of retrospective biological dosimetry could be influenced by the presence of clones, and only M-FISH can prevent clone biases.

In the case of partial body exposure, the purpose is to reconstitute the fraction of irradiated lymphocytes in order to obtain the frequency of chromosomal aberration related to the number of exposed lymphocytes. This new frequency can be converted into the dose received by the irradiated part of blood using the calibration curve. Therefore, we tried to compare the fraction of irradiated lymphocytes using the Dolphin method[33] from observations of dicentrics (fluorescence plus giemsa staining) and from stable translocation observations (M-FISH staining, data not shown). The reconstituted fraction of irradiated lymphocytes was 1/3 higher with the dicentrics than with the translocations. This is probably linked to our way of scoring. We chose to score translocations only in stable cells, *i.e.* not containing unstable aberrations, in order to simulate past exposure situations where unstable cells have disappeared.[48] But then the number of damaged cells is under estimated resulting in an underestimation of the fraction of cells exposed. Therefore, in the case of an old partial-body exposure, it is expected that whatever the FISH technique used it will be difficult to reconstitute the fraction of cells exposed. As no calibration curve was done for M-FISH, we cannot complete the dose reconstruction.

The question that remains about comparison between FISH techniques is the problem of the cost of M-FISH. M-FISH probes are much expensive than FISH-3 probes, but as the number of cells to be analyzed is smaller, fewer slides are hybridized with M-FISH than with FISH-3. The cost of reagents was therefore similar. Thus the main difference is the manpower cost, as it takes about two times longer to score metaphases with M-FISH than with FISH-3. Furthermore, unlike FISH-3, M-FISH scorers need to be well trained before being effective in chromosomal aberration analysis, which requires a longer preparation.

Conclusion

We have confirmed that M-FISH and FISH-3 do not give the same total translocation yield. First, because stable complex translocations identified by FISH-3 increased the total translocation yield. Second, because the chromosomal involvement in translocations was not that expected. Third, because the selection of scored metaphases leads to the

exclusion of more metaphases with M-FISH than with FISH-3. Finally, because clones could not be efficiently detected with FISH-3. These biases can lead to erroneous estimation of translocation yields. In the present study, FISH-3 data indicated that the translocations were stable, whereas a small decrease is observed with M-FISH. Such erroneous estimates of translocation yield could lead to wrong dose estimations in retrospective biological dosimetry. Therefore, even though M-FISH is labor-intensive, it overcomes bias inherent in FISH-3, and thereby increases the accuracy of retrospective biological dosimetry.

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