Chapter VII

In vivo validation of translocation frequency obtained by FISH with G-banding
7.1. Introduction

Cytogenetic biodosimetry originated in the mid-1960’s when chromosome aberrations were first observed by Bender in patients undergoing radiation therapy treatments. This observation led to the idea that such effects might serve as biological markers of radiation exposure. In vitro lymphocyte calibration curves were established (though not without controversy). Later in ensuing decades, the technique became firmly established when tested in a number of cases of accidental human exposure to radiation where physical data allowed dose reconstruction to provide a test of the cytogenetic biodosimeter’s usefulness.

Many in vitro generated radiation dose response curves have been utilised effectively for in vivo suspected and accidental exposures. This is evident from the many studies that have been performed using the frequencies of chromosome exchange aberrations in peripheral lymphocytes to provide useful biodosimetric information (1-4). As most accidental exposures to ionizing radiation concern partial-body irradiation, evaluation of translocation (TL) frequency in circulating lymphocytes would be useful for dose evaluation (5).

According to some published literature, the frequency of TL measured in cancer patients undergoing RT, was comparable to that observed with in vitro dose response curves (6). In vitro studies dealing with biodosimetry using TL scored by FISH and G-banding have stressed on the usefulness of FISH. An in vivo validation would be of more relevance. As TL are reliable biomarkers of radiation dose estimation, this study was carried out based on cytogenetic TL analyses (FISH and G-banding) of peripheral blood samples from cancer patients, prior to and following radiotherapy (RT), based on partial-body pelvic irradiation with Co-60 gamma rays. Hence, the present study served as an in vivo validation on the measurement of TL frequency (TL yields measured by FISH and G-banding) for cumulative dose estimation.
7.2. Methodology

Blood samples were obtained from 8 patients diagnosed with cancer (cervical / rectal / colorectal) from the Bernard Institute of Radiation Oncology, Chennai, Tamilnadu, India. About 2 ml blood was collected from each patient prior to RT and 24 hours following fractionated exposure (during or after the RT regimen). Blood collection, cytogenetic processing for FISH and G-banding was performed as detailed in Sections 2.3.1.2, 2.3.1.3, 2.3.1.5, 2.3.1.7. The details, such as age, weight, stage of tumour classification, dose to the tumour and equivalent whole body dose (EWBD) for each patient, are shown in Table 2.1. The age of the patients varied between 29 and 58 with an average of 45.75 years.

The details such as dose and dose-rate were obtained from the hospital authorities. The irradiated tissue volume necessary for EWBD estimate also was provided by the radiation physicist of the hospital. The dose delivered to the tumour during each fraction was 2 Gy. The EWBD dose to the irradiated site was calculated using the formula given by Johns (7):

\[
\text{EWBD} = \frac{\{1.44 D_o A d_{1/2} \rho [1+2.88 d_{1/2}/f]\}}{Wt}
\]

where

- \(D_o\) – Cumulative radiation dose (Gy)
- \(A\) – Field size (cm\(^2\))
- \(d_{1/2}\) – Percent depth dose (cm)
- \(\rho\) – Density = 1kg/m\(^3\)
- \(f\) – Source to skin distance (cm)
- \(Wt\) – Patient body weight (kg)

TL yields obtained for each patient by FISH and G-banding before and after therapy were analysed statistically by the paired ‘t’ test to check if they showed any significant difference. Correlation analysis was also performed to confirm the results.
7.3. Results

The results obtained for TL frequency by FISH and G-banding are shown in Tables 7.1 and 7.2 respectively. TL frequencies obtained by FISH and G-banding prior to therapy and post therapy are compared in Figures 7.1 and 7.2 respectively.

Regarding TL yields seen prior to therapy initiation, three out of eight patients demonstrated a higher TL frequency in contrast to healthy individuals, while one had a much higher frequency 0.245 TL/GE (patient 8). The TL yields in the remaining four individuals was close to the baseline frequency observed in healthy humans. Yields obtained by FISH and G-banding matched well (p = 0.435) except for 2 women (patients 3 and 8), where FISH gave higher results compared to G-banding.

The information obtained by G-banding also allowed us to check the chromosomal participation in TL formation. The profile obtained from patients before therapy is shown in Figure 7.3, while for patients after therapy, Figure 7.4 shows chromosomal involvement in TL formation. TL yields were normalized to female DNA content as given by Morton (8) and these were then used.

When compared to samples obtained before therapy, a dose dependent increase in TL yields was apparent in peripheral blood lymphocytes during / after RT. TL yields obtained by FISH and G-banding do not show any significant difference (p = 0.275). However, in two patients (patients 3 and 8), these yields differed when analysed by the 2 techniques. Figure 7.3 shows the good correlation obtained on comparison of in vivo TL yields obtained by FISH with G-banding. ($r^2 = 8260$)

Figures 7.6 and 7.7 show some interesting aberrations detected by FISH and G-banding in some individuals.
Table 7.1: Frequency and distribution of TL involving WCP 1 and WCP 3 in peripheral blood lymphocytes of cancer patients prior to therapy as resolved by FISH and G-banding.

<table>
<thead>
<tr>
<th>Cancer Type</th>
<th>Stage</th>
<th>Age (years)</th>
<th>Weight (Kg)</th>
<th>FISH</th>
<th>G Banding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CS</td>
<td>GE</td>
</tr>
<tr>
<td>1</td>
<td>Cervical</td>
<td>IIb</td>
<td>37</td>
<td>49</td>
<td>573</td>
</tr>
<tr>
<td>2</td>
<td>Cervical</td>
<td>IIb</td>
<td>35</td>
<td>57</td>
<td>500</td>
</tr>
<tr>
<td>3</td>
<td>Cervical</td>
<td>IIIb</td>
<td>52</td>
<td>67</td>
<td>160</td>
</tr>
<tr>
<td>4</td>
<td>Cervical</td>
<td>IIb</td>
<td>48</td>
<td>44</td>
<td>250</td>
</tr>
<tr>
<td>5</td>
<td>Cervical</td>
<td>Ib</td>
<td>55</td>
<td>52</td>
<td>500</td>
</tr>
<tr>
<td>6</td>
<td>Colorectal</td>
<td>-</td>
<td>29</td>
<td>47</td>
<td>405</td>
</tr>
<tr>
<td>7</td>
<td>Rectal</td>
<td>IIIc</td>
<td>58</td>
<td>55</td>
<td>176</td>
</tr>
<tr>
<td>8</td>
<td>Cervical</td>
<td>IIb</td>
<td>52</td>
<td>50</td>
<td>340</td>
</tr>
</tbody>
</table>

CS - Cells Scored  GE – Genomic Equivalent  TL – Translocation  SE – Standard Error
Validation of in vivo translocation frequency obtained by FISH with conventional G-banding

Table 7.2: Frequency and distribution of TL involving WCP 1 and WCP 3 in peripheral blood lymphocytes of cancer patients post therapy as resolved by FISH and G-banding.

<table>
<thead>
<tr>
<th>Cancer Type</th>
<th>Stage</th>
<th>Age (years)</th>
<th>Weight (kg)</th>
<th>Cum Dose (Gy)</th>
<th>Eq WBD (Gy)</th>
<th>FISH</th>
<th>G-banding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CS</td>
<td>GE</td>
</tr>
<tr>
<td>1</td>
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<td>37</td>
<td>49</td>
<td>4</td>
<td>0.5</td>
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<tr>
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<td>57</td>
<td>14</td>
<td>1.52</td>
<td>330</td>
</tr>
<tr>
<td>3</td>
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<td>IIIb</td>
<td>52</td>
<td>67</td>
<td>18</td>
<td>1.66</td>
<td>90</td>
</tr>
<tr>
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<td>48</td>
<td>44</td>
<td>16</td>
<td>2.25</td>
<td>250</td>
</tr>
<tr>
<td>5</td>
<td>Cervical</td>
<td>Ib</td>
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<td>52</td>
<td>26.4</td>
<td>3.14</td>
<td>500</td>
</tr>
<tr>
<td>6</td>
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<td>-</td>
<td>29</td>
<td>47</td>
<td>26</td>
<td>3.42</td>
<td>209</td>
</tr>
<tr>
<td>7</td>
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<td>IIIc</td>
<td>58</td>
<td>55</td>
<td>32.4</td>
<td>3.64</td>
<td>150</td>
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<tr>
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<td>IIb</td>
<td>52</td>
<td>50</td>
<td>34.6</td>
<td>4.28</td>
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</table>

<table>
<thead>
<tr>
<th>Cum Dose – Cumulative Dose</th>
<th>Eq WBD – Equivalent Whole Body Dose</th>
<th>CS – Cells Scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>GE – Genomic Equivalent</td>
<td>TL – Translocation</td>
<td>SE – Standard Error</td>
</tr>
</tbody>
</table>

Evaluation of ionizing radiation exposure for biodosimetry: Validation of Whole Chromosome Painting by Cytogenetic and Molecular techniques.
Validation of in vivo translocation frequency obtained by FISH with conventional G-banding

Figure 7.1: Comparison of translocation frequency detected by FISH and G-banding in peripheral blood lymphocytes of cancer patients prior to radiotherapy.

Figure 7.2: Comparison of translocation frequency detected by FISH and G-banding in peripheral blood lymphocytes of cancer patients post radiotherapy.
Validation of in vivo translocation frequency obtained by FISH with conventional G-banding

Figure 7.3: Profile of chromosomal participation in translocation formation observed in lymphocytes of cervical cancer patients before therapy as obtained by G-banding.

Figure 7.4: Profile of chromosomal participation in translocation formation observed in lymphocytes of cervical cancer patients after therapy as obtained by G-banding.
Figure 7.5: Comparison of translocations obtained by FISH and G-banding in peripheral blood lymphocytes from cancer patients receiving radiotherapy (0.8Gy/min Co-60 gamma radiation).

![Graph showing R^2 = 0.826](image)

Figure 7.6: Translocations observed by FISH in peripheral blood lymphocytes of cancer patients receiving radiotherapy (0.8Gy/min, Co-60 gamma radiation).
Validation of in vivo translocation frequency obtained by FISH with conventional G-banding

Evaluation of ionizing radiation exposure for biodosimetry: Validation of Whole Chromosome Painting by Cytogenetic and Molecular techniques.

Figure 7.7: Translocations recorded by G-banding in peripheral blood lymphocytes of cancer patients receiving radiotherapy (0.8Gy/min, Co-60 gamma radiation).
7.4. Discussion

We conducted a prospective study in a series of 8 patients receiving pelvic irradiation for respective cancers, to analyze TL after RT. To evaluate the frequency of TL in circulating lymphocytes, we used FISH to score TL and insertions and G-banding to validate the usefulness of FISH.

4 patients had a very low to nil rate of baseline TL, while it was elevated in the other 4 patients. It has been shown that the frequency of TL in a population is a function of age and smoking habits (9). A very low yield of TL in patients before any treatment (0.5%) was found in several other studies (10-15). However, many studies report higher frequencies of chromosomal aberration in people diagnosed with cancer when compared to healthy people. Delhanty et al., (16) found chromosomal instability in lymphocytes from patients with familial polyposis coli. Wang et al (17) determined a higher frequency of chromosomal aberrations and polymorphism in the cultured lymphocytes of 25 renal carcinoma patients than in controls. A higher frequency of cells with chromosomal alterations has been revealed by Hsu et al., (18) in the cultured peripheral blood lymphocytes of patients with multiple endocrine adenomatosis. In another series of 13 cancer patients, the basal levels of cytogenetic alterations in peripheral lymphocytes before treatment were higher than in healthy controls (16). The existence of a relationship between frequency of chromosomal aberrations in lymphocytes and the probability of the onset of cancer has been established by Bonassi et al (19) in a cohort study of a large group of 1455 subjects screened for chromosomal aberrations in Italy. The Nordic Study Group (20) and Hagman et al (21) have reported a significant increase of cancer incidence in subjects with the highest level of chromosomal aberrations.

In yet another study performed on cervical cancer patients by Murty et al (22), the frequency of metaphases with chromosome and chromatid aberrations was 17.24% in the patients while control women showed a frequency of 6.39%. There was a significant (p less than 0.001) increase in the frequency of chromosome aberrations in patients with cervical cancerous lesions, compared with controls. In our study, the higher background level of TL found in 4 patients (Patients 3, 5, 7 and 8) can be...
Validation of in vivo translocation frequency obtained by FISH with conventional G-banding

explained on the same basis - the inherent genomic instability existing in these patients (23, 24). But, we also observed nil TL yields prior to therapy, in 4 of the other individuals, part of our study. Cancer is a multi cause disease. While genomic instability could be a factor for predisposition to cancer, cervical carcinoma is also known to be caused due to certain strains of human Papilloma Virus (HPV). In such cases, genomic instability may not contribute to cancer development. This could be a possibility for the nil to very low yields observed in 4 of the patients prior to therapy.

Altogether, studying the profile of chromosomal aberrations in cancer patients prior to therapy gives one the opportunity to probe for any disease related features such as chromosomal aberrations (present study), gene mutations, etc.

With regard to TL yields studied after RT, a definite increase in yields was observed when compared to yields before initiation of therapy. We also observed inter-individual variation in the TL frequency of cancer patients irradiated with the same integral dose. On observation of yields obtained by FISH and G-banding, in one individual (patient 8), the TL yields analyzed by FISH are much higher than those obtained by G-banding. In the other patients, yields matched well between the two techniques ($r^2 = 0.826$). The reasons for this could be several. The foremost one being the easy analysis by FISH, in the observation of colourful junctions, while for G-banding, one would probe for difference in chromosome structure. Both techniques have inherent detection limitations in the size limit of chromosomal rearrangements that can be detected. The main advantage of FISH over G-banding is the chromosome morphology required. While G-banding requires excellent metaphase chromosome preparations, with FISH, one can compromise on the metaphase quality as long as it is free from cellular debris. FISH normally requires scoring of large numbers of cells. However, metaphase numbers to be scored for high doses such as those employed in our study can be brought down. While FISH is able to resolve complex TL, resolution of the same in some G-banded metaphases poses quite a challenge (25). Hence, despite the fact that G-banding allows one to score TL in every chromosome, it does have its limitations and demands of being time consuming, tedious and requirement of high quality G-banded metaphases. FISH, although being expensive makes identification and analysis of TL much simpler, faster and easy to interpret.
We were also interested to see whether TL yields gave any indication of the radioresponse of individuals in being sensitive or resistant. Most studies show that sensitivity to the induction of chromosomal damage by ionizing irradiation is higher in the lymphocytes of cancer patients than those of healthy controls. Increased chromosomal radiosensitivity is linked to cancer predisposition through mutations of various candidate genes. (26). In our study, one patient experienced severe acute toxicity to external irradiation with evident treatment-related experience, leading to the hypothesis that this toxicity might be attributed to cells with high radiosensitivity determined by genetic and/or epigenetic mechanisms. This issue is controversial. Some have attempted to correlate acute skin reactions in patients undergoing RT with results from fibroblast clonogenic assays, but such a correlation has not been established (27-28). Colony-forming assays utilizing fibroblasts are too slow; thus, leading to the use of peripheral blood lymphocytes. In a series of 48 patients treated for esophageal carcinoma, the frequency of complex-type chromosomal exchanges in blood lymphocytes correlated with acute toxicity (29). One study suggested that breast cancer patients who had severe acute skin reactions had elevated lymphocyte radiosensitivity when measured by a cytogenetic assay (30). However, Barber et al (31) did not show a significant relationship between lymphocyte radiosensitivity and normal tissue damage after RT in patients undergoing breast irradiation.

In our study, acute RT toxicity was observed in one case with observance of higher yield of TL and complex type chromosomal exchanges after radiation exposure. The cumulative radiation dose given to the patient was 4 Gy and the equivalent whole body dose was calculated to be 0.5 Gy. However, a TL frequency of 0.44 – 0.46 corresponding to 2.99-3.7Gy (extrapolated from the in vitro TL dose-response curve - FISH) was observed. On the other hand, 3 other patients – 3, 5 and 7, show extremely low TL yields when compared to their equivalent whole body doses. Investigators in other studies have observed such phenomena as well. The hormonal status of individuals, especially females, is said to play a major role in their radio-response. A number of studies on hormonal effects have been conducted. Several studies detail chromosomal fragility, breakage, and sister chromatid exchanges resulting from exposure to hormones. Various estrogens have been shown to generate significant
increases in chromatid gaps (33). Recent evidence from both in vivo and in vitro studies strongly suggests that estrogens are epigenotoxic carcinogens, i.e., they do not act directly as mutagenic or DNA-damaging agents but cause heritable changes by an unknown alternative mechanism (34). A study conducted by Roberts et al., (32), concluded that females have a greater variability in their radioreponse, and that this variability is related to progesterone, which has a profound effect upon radiosensitivity, as measured by cytogenetic end points. Prolactin and follicle-stimulating hormone also have similar effects; causing excess chromosomal gaps and breaks (35-37). These reports clearly demonstrate that a variety of female hormones may play a role in modifying or enhancing DNA damage. We could not perform in vitro irradiation of blood from these patients prior to RT, though; initial DNA damage measured on lymphocytes offers better approach in predicting the acute response of human normal tissues prior to RT (38).

Chromosomal participation in TL is a much debated topic with respect to radiation exposure. Since, we employed G-banding, we were able to obtain data for chromosomal involvement in TL formation for all chromosomes. We observed preferential involvement of the larger chromosomes in comparison to the smaller ones with few exceptions.

7.5. Conclusion

Biological dosimetry is based on the yield of chromosomal aberrations in the circulating lymphocytes and rests on the assumption that lymphocytes exposed in vivo respond in the same manner as lymphocytes exposed in vitro. The frequency of TL in human peripheral blood lymphocytes measured with the cytogenetic assay in metaphase cells has routinely been used for several decades as a tool to monitor occupational and environmental exposures to genotoxic carcinogens.

Through this study, we were able to show the usefulness and informative nature of the FISH assay even under in vivo conditions. The inclusion of the G-Banding assay helped to further validate the results obtained by FISH, showing it to be a valid and rapid method for the observance of TL.
As the trend emerging in biodosimetry is shifting from a cytogenetic observation to more personalized molecular profiling, we were interested in studying the impact of radiation at the gene level to see if radiation caused mutations in certain genes. Hence, the last section of the thesis deals with molecular analysis of \textit{in vivo} irradiated blood to analyse gene mutations.

7.6. References


