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The Applicability of Scoring Calyculin A-Induced Premature Chromosome Condensation (PCC) Objects for Dose Assessment Including for Radiotherapy Patients

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Short title: Excess PCC objects as a dosimetric endpoint

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Abstract

As an extension to a previous study, a linear calibration curve covering doses from 0 to 10 Gy was constructed and evaluated in the present study using calyculin A-induced premature chromosome condensation (PCC) by scoring excess PCC objects. The main aim of this study was to assess the applicability of this PCC assay for doses below 2 Gy that are critical for triage categorisation. Two separate blind tests involving a total of 6 doses were carried out. 4 out of 6 dose estimates were within the 95% confidence limits (95%CL) with the other 2 just outside. In addition, blood samples from five cancer patients undergoing external beam radiotherapy (RT) were also analysed and the results showed whole-body dose estimates statistically comparable to the dicentric chromosome assay (DCA) results. This is the first time that calyculin A-induced PCC was used to analyse clinical samples by scoring excess objects. Although dose estimates for the pre-RT patient samples were found to be significantly higher than the mean value for the healthy donors and were also significantly higher than those obtained using DCA, all these pre-treatment patients fell into the same category as those who may have received a low dose (< 1 Gy) and do not require immediate medical care during emergency triage. Additionally, for radiological accidents with unknown exposure scenario, PCC objects and rings can be scored in parallel for the assessment of both low and high dose exposures.

In conclusion, scoring excess objects using calyculin A-induced PCC is confirmed to be another potential biodosimetry tool in radiological emergency particularly in mass casualty scenarios even though the data need to be interpreted with caution when cancer patients are among the casualties.

Introduction

Ionising radiation, such as X-rays used in diagnostic imaging and radiotherapy (RT) as well as gamma rays and neutron particles released from nuclear weapons, can result in a wide range of direct and indirect DNA damage [IAEA 2011]. In large doses, radiation can cause serious tissue damage and increase the risk of developing cancer in later life [Clement et al. 2012]. Even for lower doses, there is no suggested threshold dose for radiation-induced malignancy based on the stochastic nature of radiation carcinogenesis [Albert 2013]. Therefore, it is critical to assess the exposure dose of the individuals as soon as possible in mass casualty radiation emergency cases. Biodosimetry, or the measurement of biological markers, such as dicentric chromosomes, translocations, micronuclei, and excess premature chromosome condensation (PCC) fragments, has proven to be a very important source of information in the evaluation of radiation overexposure; particularly, when combined with clinical signs and symptoms as well as any available physical measurement [IAEA 2011]. Dosimetric and radiological triage categorisation results are essential in the support of medical and public health decision making [Ainsbury et al. 2014].

The dicentric chromosome assay (DCA), is the current gold standard method for biological dosimetry; however, it has several inherent limitations. For example, it requires well-trained scorers, and it is not accurate for high dose exposures over 5 Gy due to cell death, mitotic delay, and the saturation of dicentrics [IAEA 2011; Pujol et al. 2014]. Calyculin A-induced PCC assay overcomes many of these limitations and has been widely used for the analysis of high dose exposures by scoring rings and excess fragments [Guerrero-Carbajal et al. 2019; Lamadrid et al. 2007; Puig et al. 2013; Romero et al. 2016] or by calculating the length ratio of the longest to the shortest chromosomes [González et al. 2014; Gotoh and Tanno 2005] or the cell cycle progression index [Miura et al. 2014]. This highly efficient and up-scalable method is particularly advantageous when there is very limited availability of blood for analysis or when metaphase spreads cannot be obtained due to very high dose exposure. Recently, scoring the number of chromosomal objects in excess of 46 in calyculin A-induced PCC has been proposed as an easy and suitable biodosimetry method in

the estimation of absorbed doses between 2 and 10 Gy [Sun et al. 2020b]. PCC objects can easily be confused with chromosomal fragments that are generated during the formation of chromosomes with multiple centromeres (e.g. dicentrics and tricentrics, etc.) and rings following exposure to high dose of radiation. They are identified as individual pieces of chromosome regardless of the shape and size and therefore eliminating the necessity to distinguish dicentrics, rings, minutes and fragments from normal chromosomes by defining each of them as one object and the excess number of objects is used as the dosimetric endpoint (Figure 1). In addition, when an exposure scenario is unknown, scoring objects and rings in parallel allows both high and low doses to be analysed using the same sets of slides or digital images [Sun et al. 2020b].

Scoring the numbers of total chromosomes at G_2 phase using calyculin A-induced PCC has been reported as a biodosimetry endpoint for low and high linear energy transfer (LET) radiation involving gamma rays [Gotoh et al. 2005] and carbon ion beam [Wang et al. 2007]. However, no specific assessment has been carried out for the suitability of this method at doses below 2 Gy; and not many cytogenetic assays have been used for the analysis of *in vivo* partial-body exposures [Darroudi et al. 1998; Hayata et al. 2001; Moquet et al. 2018; Moquet et al. 2020]. The goals for triage dosimetry are to rapidly estimate the overexposure doses, to assign the patients into the correct categories, and to provide the information for timely medical treatment [IAEA 2011]. There are three categories implemented in the MULTIBIODOSE emergency triage categorisation software [Jaworska et al. 2015]: 1. Low exposure < 1 Gy; 2. Medium exposure 1-2 Gy; 3. High exposure > 2 Gy. Therefore, dose estimation for overexposures below 2 Gy is crucial for triage categorisation.

In the present study, a calibration curve for doses between 0 and 2 Gy was constructed and the suitability for this curve to be combined with a previously published curve for doses between 2 and 10 Gy [Sun et al. 2020b] was assessed. This combined curve was validated using blind tests before it was used to evaluate the PCC method in comparison to DCA in clinical sample dose estimation. This *in vivo* study is part of the ongoing RTGene 2 project involving multi-organisations aimed at developing biomarkers of radiation response using longitudinal blood samples from cancer patients undergoing RT [Moquet et al. 2018]. The main objectives to carry out this study were to assess whether it is feasible to use calyculin A-induced PCC at 0-2 Gy for triage categorisation; and whether the results obtained using this method are comparable to those obtained from the gold standard DCA method.

Materials and Methods

All chemicals and reagents used in this project were the same as those used in a previously published study [Sun et al. 2020b]. Peripheral blood lymphocyte isolation, irradiation, and PCC induction were performed and cells at G_2 and M phases were scored as previously described [Sun et al. 2020b]. In brief, non-cycling (G_0) blood lymphocytes were isolated using Histopaque® 1077, irradiated (for calibration curve construction and blind tests, but not for patient samples) and pre-cultured with a mitogen, phytohemagglutinin (PHA), for approximately 48 hours (h) to stimulate cell division. Following irradiation, cells were kept at 37° for 2 h before PHA stimulation to allow DNA repair. Calyculin A powder was reconstituted in DMSO and subsequently diluted to working concentration (50 nM) in complete RPMI1640 medium containing 20% (v/v) foetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Calyculin A was added into the cell suspension 30 minutes (min) before harvest for PCC induction. Induced cells were finally harvested, fixed and stained for visual analysis. Written informed consent and the approval of the West Midlands-Solihull Research Ethics Committee (REC 14/WM/1182) were obtained for the healthy donors.

In the present study, isolated lymphocytes were placed into 15 mL centrifuge tubes, positioned inside a 22 mm polystyrene block with 8 mm Perspex, and sham-exposed or exposed *ex vivo* to 0, 0.25, 0.5, 0.75, 1 and 2 Gy of 250 kVp X-rays (with a half-value layer of Cu/Al filtration). The X-ray set (Ago X-ray Ltd., Martock, UK) was calibrated to a dose rate of 0.5 Gy/min and dosimetry was performed with a calibrated reference ionisation chamber for the exact exposure setup used. Exposures were always monitored using a calibrated UNIDOS E electrometer and 'in-beam' monitor ionisation chamber (all from PTW, Germany). Spatial dose uniformity was checked using Gafchromic EBT2 films (Vertec Scientific Ltd., UK). For different dose points, 200 (1 Gy), 400 (2 Gy) or 500 (0, 0.25, 0.5, 0.75 Gy) cells were scored with more scored for 2 Gy than for 1 Gy. This is because 2 Gy was the overlapping dose between the 2 separate investigations (0-2 Gy and 2-10 Gy). The blood sample from a healthy donor (female, age range 18-25 yrs) without any known previous radiation exposure was used for analysis. As a condition of the ethical approval, the actual age was not disclosed. Like dicentrics, no statistically significant inter-personal difference is believed to exist among normal non-radiosensitive individuals; therefore, no biological replicates were considered necessary for this study even though rigorous intercomparison may be needed to validate this observation. Further evidence has been referenced in a previously published study [Sun et al. 2019]. After appropriate statistical testing, the data for the above indicated irradiation doses were combined with the previously published data [Sun et al. 2020b] to generate a combined new curve covering 0-10 Gy.

Data from the previously published blind test [Sun et al. 2020b] were used to evaluate the combined calibration curve. 50 or 100 cells were scored for these three samples irradiated at higher doses (2-10 Gy). The blood sample from another donor (female; age range 45-54 yrs) was used for a fresh blind test with three additional doses. 200 cells were scored for each of these lower doses (0.2, 0.9, and 2.2 Gy).

To maintain confidentiality, coded blood samples from patients undergoing RT at Royal Marsden Hospital were sent overnight to the UK Health Security Agency (UKHSA) and were then processed in the same way as samples for the calibration curve construction without further exposure to radiation. Participants were all over the age of 18 years with (i) no previous RT (ii) no concurrent chemotherapy, hormone or biological therapy and (iii) no chemotherapy, hormone or biological therapy preceding RT by less than 4 weeks. IRAS258794 /CCR5082 RTGene 2 was approved by Wales Research Ethics Committee 7 (19/WA/0147) and registered with ClinicalTrials.gov (NCT03809377). Clinical information including tumour type, gender, prescribed target dose, number of fractions is provided in the Supplementary Table. Chemotherapy was only carried out for patient RTG-12, but not for the other four patients 4-weeks prior to RT. Age range for these five patients was 50-83 years. Two blood samples were analysed for each patient: a pre-RT control (1) and a post-RT sample (3) taken before the last radiation fraction. (1) and (3) were used to label these samples in consistence with other studies of the RTGene project. The plan was to score 200 cells for each sample; however, due to the limited availability of blood, fewer cells were scored for some of the samples i.e. one pre-RT sample: RTG-12(1), and two post-RT samples: RTG-12(3) and 13(3). In parallel, cultures for DCA were set up and processed using standard methods [IAEA 2011]. Digital images generated using the Metafer4 slide scanning system (MetaSystems, Germany) were used for scoring.

Statistics

The DoseEstimate software (version 5.1) is designed for calibration curve fitting and the calculation of doses using the statistical methods recommended by IAEA [Ainsbury and Lloyd 2010]. This software was used to calculate the mean and standard error on aberrations per cell for each dose and to fit the combined curve. Furthermore, u test and the variance to mean ratio (Var/Mean) were

used to determine whether the dispersion of excess objects followed a Poisson distribution. Values of *u* between ±1.96 are characteristic of a Poisson distribution [Papworth 1975].

The two-sample t-test was used to test for difference between the 2 Gy data point in common to both the previous 0-10 Gy and the newly established 0-2 Gy calibration curves. The paired t-test was used to compare the dose estimates between the PCC and DCA methods before and after RT treatment. A one-sample t-test was carried out to compare the mean dose estimate of the donors with the dose estimates of the pre-RT patient samples.

Results

Data used for the construction of the calibration curves generated by scoring excess objects as aberrations using calyculin A-induced PCC are shown in Table 1. The standard errors (SE) were adjusted for overdispersion for most dose points apart from 4, 6, 8 and 10 Gy. Overdispersion was also observed in one of the blind test samples (Y-2) (Table 2). Much higher degrees of overdispersion were observed in five patient samples (Table 3). For example, the highest *u* value for patient sample RTG-14(3) was 96.42; whilst for the healthy donor, the highest *u* value was 6.8.

The t-test showed no significant difference between the 2 Gy yields from the two calibration curves ($p = 0.748$), and as such it was judged possible to combine the two sets of data from the lower dose (0-2 Gy) and higher dose (0-10 Gy) calibration curves. Both a linear and a linear-quadratic calibration curve covering doses from 0 to 10 Gy were constructed and evaluated, and a linear fit to the curve was considered better in terms of more accurate dose estimation although the statistical difference between this and a linear-quadratic fit was negligible.

The combined calibration curve was fitted to a linear model: $Y= 0.0433 (+/- 0.0182) + 0.6970 (+/- 0.0584)*D$, in which Y represents the yield of excess objects, and D the dose (Figure 2). For this curve, the p value for goodness of fit was < 0.0001, and the p values for coefficients (z-test) were: $p\_A = 0.0446$, and $p\_alpha < 0.0001$. The correlation coefficient of the curve was: $r = 0.9959$. Calculated using this curve, 4 out of 6 dose estimates were within the 95% confidence limits (95%CL) with the other 2 just outside (Table 2) in the blind validation tests.

Blood samples from five cancer patients were also assessed to give a dose estimate using this combined calibration curve. Results showed that the dose estimates for the pre-treatment patient samples were significantly higher than the mean value for the healthy donors (mean = 0.032 Gy, $p = 0.035$); and were also significantly ($p = 0.048$) higher than those obtained using DCA (Table 4). For the post-RT patient samples (Table 5), the t-test comparison for the dose estimates generated using PCC showed no significant difference to the DCA data ($p = 0.406$) overall within the group of five patient samples. Importantly, our results also showed that the whole-body dose estimates for all five cancer patients before RT were below 1 Gy; and therefore, these patients all fell into the same triage category as those with low dose exposure and do not require urgent treatment (Table 4).

Discussions and Conclusions

Radiological emergencies involving nuclear power plant accidents, the use of nuclear weapons or terrorist attacks can result in mass casualty situations whereby a large number of individuals are exposed or are suspected to have been exposed. The estimation of the radiation dose of potentially exposed individuals using cytogenetic approaches can assist health workers to quickly triage those who require urgent medical treatment and/or monitoring for longer term health effects from those who are not at risk. As a well-established method in biodosimetry, DCA is more accurate for doses below 2 Gy than the calyculin A-induced PCC. PCC is therefore not preferred for this dose range, but

rather it is suggested as an alternative method especially when it is difficult to obtain sufficient number of cells to score for the elderly and those with pathological conditions [Gotoh and Durante 2006; Hatzi et al. 2006; M'Kacher et al. 2023]. Using calyculin A, PCC can be induced with high efficiency in terms of a much higher number of cells to score in comparison to DCA using the same amount of blood [Sun et al. 2020a].

A high level of overdispersion was seen in all five cancer patient samples suggesting a partial-body nature of exposure even though overdispersion was also observed in some of the samples for calibration curve fitting and one in the blind evaluation tests. The cause for the overdispersion of excess objects in the blood of healthy volunteers is unclear at present. However, overdispersion for the distribution of excess acentrics among cells is common [Cornforth and Goodwin 1991; Schmid and Bauchinger 1980; Virsik and Harder 1981]. Virsik and Harder [Virsik and Harder 1981] suggested an aberration mechanism in which overdispersion of acentrics occurs when more than one acentric is formed simultaneously. Cornforth and Goodwin [Cornforth and Goodwin 1991] suggested that overdispersion appears to be a general feature of high LET (e.g. 238Pu alpha-particle) radiation induced PCC fragmentation assuming that single particle traversals are capable of producing multiple fragments. As discussed below, the formation of excess PCC objects can result from multiple factors; and therefore, it is highly likely that the aberration mechanism can result in simultaneous formation of more than one object in the cell and thus cause overdispersion. It should be noted that high LET radiation is mainly referenced to assist with the explanation as X-rays are low LET radiation.

Blood is constantly circulating in the body and only a small proportion of the blood cell population is exposed to the external beam in certain selected areas for the individual treatment fraction. It is possible that some cells are hit by the beam more than once and sustain heavy damage to the genetic material. Therefore, there is a very small number of heavily damaged lymphocytes randomly distributed in the blood of the partially exposed patients, which could partly explain the overdispersion of PCC objects in the patient samples. Similarly, overdispersion of dicentrics was also observed in DCA results. In addition, it is possible that damaged T-lymphocytes may reside in the lymph nodes in the treatment field during several fractions and then enter the circulation, which may subsequently contribute to overdispersion.

The background frequency of excess objects for calyculin A-induced PCC is higher than the background level of dicentrics in DCA. A background level of approximately 4-6% excess fragments has been reported [Balakrishnan et al. 2010; Puig et al. 2013; Sun et al. 2020b] for chemical induced PCC. In comparison, the spontaneous incidence of dicentrics is approximately 0-2 in 1000 cells [IAEA 2011]. Dicentric chromosomes are rare events as the result of mis-repaired DNA double strand breaks [IAEA 2011]; whilst chemicals such as calyculin A, aphidicolin [Achkar et al. 2005], and bleomycin [Bolzán and Bianchi 2018] can all induce single- and double-strand breaks in DNA and may subsequently lead to the formation of PCC fragments. Exposure to environmental clastogens and aneugens (increasing with age) can also lead to the fragmentation of chromosomes [Alhmoud et al. 2020]. Hitherto, no population study has been carried out to assess the effects of other biological or environmental factors on PCC fragmentation, such as age, alcohol intake, smoking status and occupational hazards. Clinical treatments (e.g. chemotherapy and CT scan) may also cause the formation of excess PCC fragments. As most mass casualty accidents will be caused by gamma rays, a future study assessing the effects of gamma radiation on the number of excess PCC objects would also be beneficial.

Importantly, the numbers of excess PCC objects for the pre-RT patient samples (Table 4) were found to be much higher than the mean value for the healthy donors. The higher pre-RT frequency of PCC objects in the cancer patients may be attributable to age (mean=71 years). It is also possible that the

patients involved in this study had been exposed to chemotherapy as well as repeated diagnostic radiology over 4 weeks prior to RT, such as CT scans and PET-CTs. Another plausible cause for this difference is that calyculin A induces chromosome damage at common fragile sites (CFSs) after perturbation of the replication dynamics [Achkar et al. 2005]. CFSs instability could be responsible for chromosome rearrangements and are frequently correlated with cancers [Glover et al. 2017; Ma et al. 2012]. Our results suggest that the CFSs of cancer patients may be more prone to calyculin A-induced breaks than healthy donors. It would be worthwhile to investigate the effect of calyculin A on the alteration of CFSs in cancer patients. Our analysis found that dose estimates for the post-RT cancer patient samples were statistically comparable to those from DCA. However, sample RTG-25(3) showed an unexpectedly higher dose estimate. The pre-RT sample for this patient, RTG-25(1), also showed a higher level of excess objects. Because this patient did not have any chemo/biological treatment 4-weeks prior to RT, it is possible that she may have genomic instability associated with CFSs, which manifested as an increased number of chromosomal breaks in the PCC inducing procedure.

For triage dosimetry, the goal is to assign the patients with suspected overexposure into the appropriate category quickly and correctly to advise on medical interventions. In the present study, the whole-body dose estimates for all five cancer patients prior to RT were found to be below 1 Gy, and thus can be allocated into the same triage category as those who have received a small dose but do not need urgent treatment. Therefore, calyculin A-induced PCC can potentially serve the purpose for triage categorisation in mass casualty accidents or terrorist attacks, but further work will be needed. Even though it is beyond the scope of the present study, further information may need to be included in future studies with valid control samples, such as the type and stage of cancer, the type of irradiation facility, the dose used in each RT fraction as well as the gap between fractions as these may have significant impact on dose estimation.

In conclusion, the point of introducing this biodosimetry method is to eliminate the time-consuming identification of different types of aberrations so that the scoring can potentially be done by inexperienced workers in case of large-scale nuclear emergency. The simplicity in scoring may also enable the automation of the scoring procedure. Further work will be required to understand the issue of overdispersion as well as individual variability in background samples with age and other confounding factors taken into consideration. For unexposed cancer patients in similar circumstances, this assay may not be applicable to identify these individuals in radiation emergencies. However, in this exploratory study it has been demonstrated that PCC is a valuable approach with the potential to complement or be used as an alternative to the DCA. Particularly, owing to its high induction efficiency for scorable cells, PCC can potentially be applied when the availability of blood is extremely limited, or when the suspected overexposure is higher than 5 Gy and the DCA method may fail to produce sufficient metaphase spreads for analysis.

Statements

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Statement of Ethics

The research was conducted in compliance with internationally accepted ethical standards for research practice and reporting. Written informed consent and the approval of the West Midlands-Solihull Research Ethics Committee (REC 14/WM/1182) were obtained for the healthy donors. The

clinical trial, IRAS258794 /CCR5082 RTGene 2, was approved by Wales Research Ethics Committee 7 (19/WA/0147) and registered with ClinicalTrials.gov (NCT03809377).

Conflict of Interest Statement

The views expressed are those of the authors and not necessarily those of the NIHR, the Department of Health or UKHSA. The authors have no relevant financial or non-financial interests to disclose. The authors have no competing interests to declare that are relevant to the content of this article.

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Author Contributions

Elizabeth Ainsbury, Navita Somaiah, and Jayne Moquet contributed to the funding application and the setting up of the RTGene 2 project.
Mingzhu Sun and Jayne Moquet conceived of the presented idea and performed the analytic calculations.
Jayne Moquet supervised the findings of this work and carried out the experiments using DCA.
Mingzhu Sun carried out the experiments using PCC and wrote the manuscript with support from all authors.
Elizabeth Ainsbury verified the analytical methods and contributed to the interpretation of the results.
Selvakumar Anbalagan, Harriet Steel, Aurore Sommer, and Lone Gothard contributed to patient recruitment, sample collection and transport.
David Lloyd provided critical review for the draft manuscript.
Stephen Barnard provided technical assistance for the project.
All authors provided critical feedback to help shape the research and analysis. All authors discussed the results and contributed to the final version of the manuscript.

Data Availability Statement

All datasets on which the conclusions of the paper rely are available to editors, reviewers and readers without unnecessary restriction wherever possible. All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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Figure legend:

Figure 1. One Giemsa-stained G_2 phase PCC cell containing 55 objects with 9 excess above 46 (irradiated at 10 Gy). Every individual chromosomal piece regardless of shape and size is scored as one object. Numbers in red are placed next to the objects to assist with the understanding of the scoring. The blood donor was a healthy male aged in the range of 25-34 yrs.

Figure 2. The combined calibration curve covering 0-10 Gy was fitted to a linear model: Y= 0.0433 (+/- 0.0182) + 0.6970 (+/- 0.0584)*D, in which Y represents the yield of excess objects, and D the dose. Bars represent standard error.

The Applicability of Scoring Calyculin A-Induced Premature Chromosome Condensation (PCC) Objects for Dose Assessment Including for Radiotherapy Patients

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Abstract

As an extension to a previous study, a linear calibration curve covering doses from 0 to 10 Gy was constructed and evaluated in the present study using calyculin A-induced premature chromosome condensation (PCC) by scoring excess PCC objects. The main aim of this study was to assess the applicability of this PCC assay for doses below 2 Gy that are critical for triage categorisation. Two separate blind tests involving a total of 6 doses were carried out. 4 out of 6 dose estimates were within the 95% confidence limits (95%CL) with the other 2 just outside. In addition, blood samples from five cancer patients undergoing external beam radiotherapy (RT) were also analysed and the results showed whole-body dose estimates statistically comparable to the dicentric chromosome assay (DCA) results. This is the first time that calyculin A-induced PCC was used to analyse clinical samples by scoring excess objects. Although dose estimates for the pre-RT patient samples were found to be significantly higher than the mean value for the healthy donors and were also significantly higher than those obtained using DCA, all these pre-treatment patients fell into the same category as those who may have received a low dose (< 1 Gy) and do not require immediate medical care during emergency triage. Additionally, for radiological accidents with unknown exposure scenario, PCC objects and rings can be scored in parallel for the assessment of both low and high dose exposures.

In conclusion, scoring excess objects using calyculin A-induced PCC is confirmed to be another potential biodosimetry tool in radiological emergency particularly in mass casualty scenarios even though the data need to be interpreted with caution when cancer patients are among the casualties.

Introduction

Ionising radiation, such as X-rays used in diagnostic imaging and radiotherapy (RT) as well as gamma rays and neutron particles released from nuclear weapons, can result in a wide range of direct and indirect DNA damage [IAEA 2011]. In large doses, radiation can cause serious tissue damage and increase the risk of developing cancer in later life [Clement et al. 2012]. Even for lower doses, there is no suggested threshold dose for radiation-induced malignancy based on the stochastic nature of radiation carcinogenesis [Albert 2013]. Therefore, it is critical to assess the exposure dose of the individuals as soon as possible in mass casualty radiation emergency cases. Biodosimetry, or the measurement of biological markers, such as dicentric chromosomes, translocations, micronuclei, and excess premature chromosome condensation (PCC) fragments, has proven to be a very important source of information in the evaluation of radiation overexposure; particularly, when combined with clinical signs and symptoms as well as any available physical measurement [IAEA 2011]. Dosimetric and radiological triage categorisation results are essential in the support of medical and public health decision making [Ainsbury et al. 2014].

The dicentric chromosome assay (DCA), is the current gold standard method for biological dosimetry; however, it has several inherent limitations. For example, it requires well-trained scorers, and it is not accurate for high dose exposures over 5 Gy due to cell death, mitotic delay, and the saturation of dicentrics [IAEA 2011; Pujol et al. 2014]. Calyculin A-induced PCC assay overcomes many of these limitations and has been widely used for the analysis of high dose exposures by scoring rings and excess fragments [Guerrero-Carbajal et al. 2019; Lamadrid et al. 2007; Puig et al. 2013; Romero et al. 2016] or by calculating the length ratio of the longest to the shortest chromosomes [González et al. 2014; Gotoh and Tanno 2005] or the cell cycle progression index [Miura et al. 2014]. This highly efficient and up-scalable method is particularly advantageous when there is very limited availability of blood for analysis or when metaphase spreads cannot be obtained due to very high dose exposure. Recently, scoring the number of chromosomal objects in excess of 46 in calyculin A-induced PCC has been proposed as an easy and suitable biodosimetry method in

the estimation of absorbed doses between 2 and 10 Gy [Sun et al. 2020b]. PCC objects can easily be confused with chromosomal fragments that are generated during the formation of chromosomes with multiple centromeres (e.g. dicentrics and tricentrics, etc.) and rings following exposure to high dose of radiation. They are identified as individual pieces of chromosome regardless of the shape and size and therefore eliminating the necessity to distinguish dicentrics, rings, minutes and fragments from normal chromosomes by defining each of them as one object and the excess number of objects is used as the dosimetric endpoint (Figure 1). In addition, when an exposure scenario is unknown, scoring objects and rings in parallel allows both high and low doses to be analysed using the same sets of slides or digital images [Sun et al. 2020b].

Scoring the numbers of total chromosomes at G_2 phase using calyculin A-induced PCC has been reported as a biodosimetry endpoint for low and high linear energy transfer (LET) radiation involving gamma rays [Gotoh et al. 2005] and carbon ion beam [Wang et al. 2007]. However, no specific assessment has been carried out for the suitability of this method at doses below 2 Gy; and not many cytogenetic assays have been used for the analysis of *in vivo* partial-body exposures [Darroudi et al. 1998; Hayata et al. 2001; Moquet et al. 2018; Moquet et al. 2020]. The goals for triage dosimetry are to rapidly estimate the overexposure doses, to assign the patients into the correct categories, and to provide the information for timely medical treatment [IAEA 2011]. There are three categories implemented in the MULTIBIODOSE emergency triage categorisation software [Jaworska et al. 2015]: 1. Low exposure < 1 Gy; 2. Medium exposure 1-2 Gy; 3. High exposure > 2 Gy. Therefore, dose estimation for overexposures below 2 Gy is crucial for triage categorisation.

In the present study, a calibration curve for doses between 0 and 2 Gy was constructed and the suitability for this curve to be combined with a previously published curve for doses between 2 and 10 Gy [Sun et al. 2020b] was assessed. This combined curve was validated using blind tests before it was used to evaluate the PCC method in comparison to DCA in clinical sample dose estimation. This

in vivo study is part of the ongoing RTGene 2 project involving multi-organisations aimed at developing biomarkers of radiation response using longitudinal blood samples from cancer patients undergoing RT [Moquet et al. 2018]. The main objectives to carry out this study were to assess whether it is feasible to use calyculin A-induced PCC at 0-2 Gy for triage categorisation; and whether the results obtained using this method are comparable to those obtained from the gold standard DCA method.

Materials and Methods

All chemicals and reagents used in this project were the same as those used in a previously published study [Sun et al. 2020b]. Peripheral blood lymphocyte isolation, irradiation, and PCC induction were performed and cells at G_2 and M phases were scored as previously described [Sun et al. 2020b]. In brief, non-cycling (G_0) blood lymphocytes were isolated using Histopaque® 1077, irradiated (for calibration curve construction and blind tests, but not for patient samples) and pre-cultured with a mitogen, phytohemagglutinin (PHA), for approximately 48 hours (h) to stimulate cell division. Following irradiation, cells were kept at 37° for 2 h before PHA stimulation to allow DNA repair. Calyculin A powder was reconstituted in DMSO and subsequently diluted to working concentration (50 nM) in complete RPMI1640 medium containing 20% (v/v) foetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. Calyculin A was added into the cell suspension 30 minutes (min) before harvest for PCC induction. Induced cells were finally harvested, fixed and stained for visual analysis. Written informed consent and the approval of the West Midlands-Solihull Research Ethics Committee (REC 14/WM/1182) were obtained for the healthy donors.

In the present study, isolated lymphocytes were placed into 15 mL centrifuge tubes, positioned inside a 22 mm polystyrene block with 8 mm Perspex, and sham-exposed or exposed *ex vivo* to 0,

0.25, 0.5, 0.75, 1 and 2 Gy of 250 kVp X-rays (with a half-value layer of Cu/Al filtration). The X-ray set (Ago X-ray Ltd., Martock, UK) was calibrated to a dose rate of 0.5 Gy/min and dosimetry was performed with a calibrated reference ionisation chamber for the exact exposure setup used. Exposures were always monitored using a calibrated UNIDOS E electrometer and 'in-beam' monitor ionisation chamber (all from PTW, Germany). Spatial dose uniformity was checked using Gafchromic EBT2 films (Vertec Scientific Ltd., UK). For different dose points, 200 (1 Gy), 400 (2 Gy) or 500 (0, 0.25, 0.5, 0.75 Gy) cells were scored with more scored for 2 Gy than for 1 Gy. This is because 2 Gy was the overlapping dose between the 2 separate investigations (0-2 Gy and 2-10 Gy). The blood sample from a healthy donor (female, age range 18-25 yrs) without any known previous radiation exposure was used for analysis. As a condition of the ethical approval, the actual age was not disclosed. Like dicentrics, no statistically significant inter-personal difference is believed to exist among normal non-radiosensitive individuals; therefore, no biological replicates were considered necessary for this study even though rigorous intercomparison may be needed to validate this observation. Further evidence has been referenced in a previously published study [Sun et al. 2019]. After appropriate statistical testing, the data for the above indicated irradiation doses were combined with the previously published data [Sun et al. 2020b] to generate a combined new curve covering 0-10 Gy.

Data from the previously published blind test [Sun et al. 2020b] were used to evaluate the combined calibration curve. 50 or 100 cells were scored for these three samples irradiated at higher doses (2-10 Gy). The blood sample from another donor (female; age range 45-54 yrs) was used for a fresh blind test with three additional doses. 200 cells were scored for each of these lower doses (0.2, 0.9, and 2.2 Gy).

To maintain confidentiality, coded blood samples from patients undergoing RT at Royal Marsden Hospital were sent overnight to the UK Health Security Agency (UKHSA) and were then processed in

the same way as samples for the calibration curve construction without further exposure to radiation. Participants were all over the age of 18 years with (i) no previous RT (ii) no concurrent chemotherapy, hormone or biological therapy and (iii) no chemotherapy, hormone or biological therapy preceding RT by less than 4 weeks. IRAS258794 /CCR5082 RTGene 2 was approved by Wales Research Ethics Committee 7 (19/WA/0147) and registered with ClinicalTrials.gov (NCT03809377). Clinical information including tumour type, gender, prescribed target dose, number of fractions is provided in the Supplementary Table. Chemotherapy was only carried out for patient RTG-12, but not for the other four patients 4-weeks prior to RT. Age range for these five patients was 50-83 years. Two blood samples were analysed for each patient: a pre-RT control (1) and a post-RT sample (3) taken before the last radiation fraction. (1) and (3) were used to label these samples in consistence with other studies of the RTGene project. The plan was to score 200 cells for each sample; however, due to the limited availability of blood, fewer cells were scored for some of the samples i.e. one pre-RT sample: RTG-12(1), and two post-RT samples: RTG-12(3) and 13(3). In parallel, cultures for DCA were set up and processed using standard methods [IAEA 2011]. Digital images generated using the Metafer4 slide scanning system (MetaSystems, Germany) were used for scoring.

Statistics

The DoseEstimate software (version 5.1) is designed for calibration curve fitting and the calculation of doses using the statistical methods recommended by IAEA [Ainsbury and Lloyd 2010]. This software was used to calculate the mean and standard error on aberrations per cell for each dose and to fit the combined curve. Furthermore, u test and the variance to mean ratio (Var/Mean) were used to determine whether the dispersion of excess objects followed a Poisson distribution. Values of u between ±1.96 are characteristic of a Poisson distribution [Papworth 1975].

The two-sample t-test was used to test for difference between the 2 Gy data point in common to both the previous 0-10 Gy and the newly established 0-2 Gy calibration curves. The paired t-test was used to compare the dose estimates between the PCC and DCA methods before and after RT treatment. A one-sample t-test was carried out to compare the mean dose estimate of the donors with the dose estimates of the pre-RT patient samples.

Results

Data used for the construction of the calibration curves generated by scoring excess objects as aberrations using calyculin A-induced PCC are shown in Table 1. The standard errors (SE) were adjusted for overdispersion for most dose points apart from 4, 6, 8 and 10 Gy. Overdispersion was also observed in one of the blind test samples (Y-2) (Table 2). Much higher degrees of overdispersion were observed in five patient samples (Table 3). For example, the highest *u* value for patient sample RTG-14(3) was 96.42; whilst for the healthy donor, the highest *u* value was 6.8.

The t-test showed no significant difference between the 2 Gy yields from the two calibration curves ($p = 0.748$), and as such it was judged possible to combine the two sets of data from the lower dose (0-2 Gy) and higher dose (0-10 Gy) calibration curves. Both a linear and a linear-quadratic calibration curve covering doses from 0 to 10 Gy were constructed and evaluated, and a linear fit to the curve was considered better in terms of more accurate dose estimation although the statistical difference between this and a linear-quadratic fit was negligible.

The combined calibration curve was fitted to a linear model: $Y = 0.0433 (+/- 0.0182) + 0.6970 (+/- 0.0584) * D$, in which Y represents the yield of excess objects, and D the dose (Figure 2). For this curve, the p value for goodness of fit was < 0.0001, and the p values for coefficients (z-test) were: $p\_A = 0.0446$, and $p\_alpha < 0.0001$. The correlation coefficient of the curve was: $r = 0.9959$.

Calculated using this curve, 4 out of 6 dose estimates were within the 95% confidence limits (95%CL) with the other 2 just outside (Table 2) in the blind validation tests.

Blood samples from five cancer patients were also assessed to give a dose estimate using this combined calibration curve. Results showed that the dose estimates for the pre-treatment patient samples were significantly higher than the mean value for the healthy donors (mean = 0.032 Gy, $p = 0.035$); and were also significantly ($p = 0.048$) higher than those obtained using DCA (Table 4). For the post-RT patient samples (Table 5), the t-test comparison for the dose estimates generated using PCC showed no significant difference to the DCA data ($p = 0.406$) overall within the group of five patient samples. Importantly, our results also showed that the whole-body dose estimates for all five cancer patients before RT were below 1 Gy; and therefore, these patients all fell into the same triage category as those with low dose exposure and do not require urgent treatment (Table 4).

Discussions and Conclusions

Radiological emergencies involving nuclear power plant accidents, the use of nuclear weapons or terrorist attacks can result in mass casualty situations whereby a large number of individuals are exposed or are suspected to have been exposed. The estimation of the radiation dose of potentially exposed individuals using cytogenetic approaches can assist health workers to quickly triage those who require urgent medical treatment and/or monitoring for longer term health effects from those who are not at risk. As a well-established method in biodosimetry, DCA is more accurate for doses below 2 Gy than the calyculin A-induced PCC. PCC is therefore not preferred for this dose range, but rather it is suggested as an alternative method especially when it is difficult to obtain sufficient number of cells to score for the elderly and those with pathological conditions [Gotoh and Durante 2006; Hatzi et al. 2006; M'Kacher et al. 2023]. Using calyculin A, PCC can be induced with high

efficiency in terms of a much higher number of cells to score in comparison to DCA using the same amount of blood [Sun et al. 2020a].

A high level of overdispersion was seen in all five cancer patient samples suggesting a partial-body nature of exposure even though overdispersion was also observed in some of the samples for calibration curve fitting and one in the blind evaluation tests. The cause for the overdispersion of excess objects in the blood of healthy volunteers is unclear at present. However, overdispersion for the distribution of excess acentrics among cells is common [Cornforth and Goodwin 1991; Schmid and Bauchinger 1980; Virsik and Harder 1981]. Virsik and Harder [Virsik and Harder 1981] suggested an aberration mechanism in which overdispersion of acentrics occurs when more than one acentric is formed simultaneously. Cornforth and Goodwin [Cornforth and Goodwin 1991] suggested that overdispersion appears to be a general feature of high LET (e.g. 238Pu alpha-particle) radiation induced PCC fragmentation assuming that single particle traversals are capable of producing multiple fragments. As discussed below, the formation of excess PCC objects can result from multiple factors; and therefore, it is highly likely that the aberration mechanism can result in simultaneous formation of more than one object in the cell and thus cause overdispersion. It should be noted that high LET radiation is mainly referenced to assist with the explanation as X-rays are low LET radiation.

Blood is constantly circulating in the body and only a small proportion of the blood cell population is exposed to the external beam in certain selected areas for the individual treatment fraction. It is possible that some cells are hit by the beam more than once and sustain heavy damage to the genetic material. Therefore, there is a very small number of heavily damaged lymphocytes randomly distributed in the blood of the partially exposed patients, which could partly explain the overdispersion of PCC objects in the patient samples. Similarly, overdispersion of dicentrics was also observed in DCA results. In addition, it is possible that damaged T-lymphocytes may reside in the

lymph nodes in the treatment field during several fractions and then enter the circulation, which may subsequently contribute to overdispersion.

The background frequency of excess objects for calyculin A-induced PCC is higher than the background level of dicentrics in DCA. A background level of approximately 4-6% excess fragments has been reported [Balakrishnan et al. 2010; Puig et al. 2013; Sun et al. 2020b] for chemical induced PCC. In comparison, the spontaneous incidence of dicentrics is approximately 0-2 in 1000 cells [IAEA 2011]. Dicentric chromosomes are rare events as the result of mis-repaired DNA double strand breaks [IAEA 2011]; whilst chemicals such as calyculin A, aphidicolin [Achkar et al. 2005], and bleomycin [Bolzán and Bianchi 2018] can all induce single- and double-strand breaks in DNA and may subsequently lead to the formation of PCC fragments. Exposure to environmental clastogens and aneugens (increasing with age) can also lead to the fragmentation of chromosomes [Alhmoud et al. 2020]. Hitherto, no population study has been carried out to assess the effects of other biological or environmental factors on PCC fragmentation, such as age, alcohol intake, smoking status and occupational hazards. Clinical treatments (e.g. chemotherapy and CT scan) may also cause the formation of excess PCC fragments. As most mass casualty accidents will be caused by gamma rays, a future study assessing the effects of gamma radiation on the number of excess PCC objects would also be beneficial.

Importantly, the numbers of excess PCC objects for the pre-RT patient samples (Table 4) were found to be much higher than the mean value for the healthy donors. The higher pre-RT frequency of PCC objects in the cancer patients may be attributable to age (mean=71 years). It is also possible that the patients involved in this study had been exposed to chemotherapy as well as repeated diagnostic radiology over 4 weeks prior to RT, such as CT scans and PET-CTs. Another plausible cause for this difference is that calyculin A induces chromosome damage at common fragile sites (CFSs) after perturbation of the replication dynamics [Achkar et al. 2005]. CFSs instability could be responsible

for chromosome rearrangements and are frequently correlated with cancers [Glover et al. 2017; Ma et al. 2012]. Our results suggest that the CFSs of cancer patients may be more prone to calyculin A-induced breaks than healthy donors. It would be worthwhile to investigate the effect of calyculin A on the alteration of CFSs in cancer patients. Our analysis found that dose estimates for the post-RT cancer patient samples were statistically comparable to those from DCA. However, sample RTG-25(3) showed an unexpectedly higher dose estimate. The pre-RT sample for this patient, RTG-25(1), also showed a higher level of excess objects. Because this patient did not have any chemo/biological treatment 4-weeks prior to RT, it is possible that she may have genomic instability associated with CFSs, which manifested as an increased number of chromosomal breaks in the PCC inducing procedure.

For triage dosimetry, the goal is to assign the patients with suspected overexposure into the appropriate category quickly and correctly to advise on medical interventions. In the present study, the whole-body dose estimates for all five cancer patients prior to RT were found to be below 1 Gy, and thus can be allocated into the same triage category as those who have received a small dose but do not need urgent treatment. Therefore, calyculin A-induced PCC can potentially serve the purpose for triage categorisation in mass casualty accidents or terrorist attacks, but further work will be needed. Even though it is beyond the scope of the present study, further information may need to be included in future studies with valid control samples, such as the type and stage of cancer, the type of irradiation facility, the dose used in each RT fraction as well as the gap between fractions as these may have significant impact on dose estimation.

In conclusion, the point of introducing this biodosimetry method is to eliminate the time-consuming identification of different types of aberrations so that the scoring can potentially be done by inexperienced workers in case of large-scale nuclear emergency. The simplicity in scoring may also enable the automation of the scoring procedure. Further work will be required to understand the

issue of overdispersion as well as individual variability in background samples with age and other confounding factors taken into consideration. For unexposed cancer patients in similar circumstances, this assay may not be applicable to identify these individuals in radiation emergencies. However, in this exploratory study it has been demonstrated that PCC is a valuable approach with the potential to complement or be used as an alternative to the DCA. Particularly, owing to its high induction efficiency for scorable cells, PCC can potentially be applied when the availability of blood is extremely limited, or when the suspected overexposure is higher than 5 Gy and the DCA method may fail to produce sufficient metaphase spreads for analysis.

Statements

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Statement of Ethics

The research was conducted in compliance with internationally accepted ethical standards for research practice and reporting. Written informed consent and the approval of the West Midlands-Solihull Research Ethics Committee (REC 14/WM/1182) were obtained for the healthy donors. The clinical trial, IRAS258794 /CCR5082 RTGene 2, was approved by Wales Research Ethics Committee 7 (19/WA/0147) and registered with ClinicalTrials.gov (NCT03809377).

Conflict of Interest Statement

The views expressed are those of the authors and not necessarily those of the NIHR, the Department of Health or UKHSA. The authors have no relevant financial or non-financial interests to disclose. The authors have no competing interests to declare that are relevant to the content of this article.

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Author Contributions

Elizabeth Ainsbury, Navita Somaiah, and Jayne Moquet contributed to the funding application and the setting up of the RTGene 2 project.

Mingzhu Sun and Jayne Moquet conceived of the presented idea and performed the analytic calculations.

Jayne Moquet supervised the findings of this work and carried out the experiments using DCA.

Mingzhu Sun carried out the experiments using PCC and wrote the manuscript with support from all authors.

Elizabeth Ainsbury verified the analytical methods and contributed to the interpretation of the results.

Selvakumar Anbalagan, Harriet Steel, Aurore Sommer, and Lone Gothard contributed to patient recruitment, sample collection and transport.

David Lloyd provided critical review for the draft manuscript.

Stephen Barnard provided technical assistance for the project.

All authors provided critical feedback to help shape the research and analysis. All authors discussed the results and contributed to the final version of the manuscript.

Data Availability Statement

All datasets on which the conclusions of the paper rely are available to editors, reviewers and readers without unnecessary restriction wherever possible. All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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Figure legend:

Figure 1. One Giemsa-stained G_2 phase PCC cell containing 55 objects with 9 excess above 46 (irradiated at 10 Gy). Every individual chromosomal piece regardless of shape and size is scored as one object. Numbers in red are placed next to the objects to assist with the understanding of the scoring. The blood donor was a healthy male aged in the range of 25-34 yrs.

Figure 2. The combined calibration curve covering 0-10 Gy was fitted to a linear model: Y= 0.0433 (+/- 0.0182) + 0.6970 (+/- 0.0584)*D, in which Y represents the yield of excess objects, and D the dose. Bars represent standard error.


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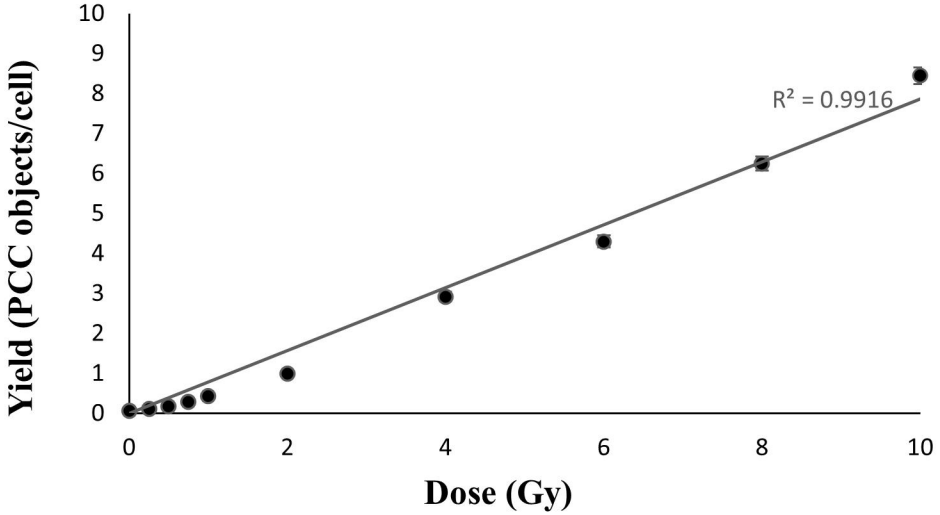
Yield (PCC objects/cell)
Dose (Gy)
R² = 0.9916
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Table 1. Data generated for the construction of the calibration curve (Figure 2) using calyculin A-induced PCC by scoring excess objects.

Dose (Gy)	Cells Scored	Aberrations	Distribution of Excess Objects																				Yield±SE	Var/Mean±SE	u
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20			
0	1500	98	1410	82	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.065±0.008	1.1±0.036	2.72
0.25	500	56	453	41	5	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.112±0.017	1.43±0.063	6.8
0.5	500	89	431	53	13	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.178±0.022	1.39±0.063	6.14
0.75	500	146	388	84	22	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.292±0.028	1.26±0.063	4.1
1	200	87	140	35	23	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.435±0.055	1.24±0.1	2.39
2	600	600	256	178	104	42	13	6	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1±0.048	1.28±0.058	4.83
4	200	584	17	23	47	50	28	19	7	6	1	2	0	0	0	0	0	0	0	0	0	0	0	2.92±0.14	1.12±0.1	1.2
6	200	861	3	9	27	30	46	31	29	11	10	2	2	0	0	0	0	0	0	0	0	0	0	4.3±0.147	0.906±0.1	-0.936
8	200	1247	0	1	6	12	25	37	33	33	18	19	10	3	3	0	0	0	0	0	0	0	0	6.24±0.177	0.785±0.1	-2.15
10	200	1687	0	1	1	2	8	18	21	31	37	17	19	20	8	5	3	4	1	1	0	2	1	8.44±0.238	1.11±0.1	1.1

Aberrations were scored as the total numbers of excess PCC objects at G_2 and M phases. SE=Standard Error. For 0 and 2 Gy, the numbers of cells scored at two dose ranges (0-2Gy and 0-10Gy) in two separate studies were combined to get 1500 and 600 cells in total, respectively. No other dose was scored at two dose ranges.

Table 2. Dose estimation and distribution of excess PCC objects in the blind tests.

Sample ID (Blind Tests)	No. of Cells Scored	No. of Aberrations	Actual Dose (Gy)	Dose Estimate (Gy)	Lower 95% CL	Upper 95% CL	Distribution of Excess Objects														Yield±SE	Var/Mean±SE	u
							0	1	2	3	4	5	6	7	8	9	10	11	12	13	14			
X-1	100	156	2.4	2.176 ± 0.180	1.824	2.528	19	34	27	12	8											1.56 ± 0.125	0.872 ± 0.142	-0.905
Y-1	50	370	9.2	10.550 ± 0.552	9.473	11.64	0	0	1	2	5	5	6	7	7	4	9	1	1	1	1	7.4 ± 0.385	0.965 ± 0.202	-0.172
Z-1	50	215	5.6	6.107 ± 0.421	5.282	6.932	1	6	6	9	6	7	6	4	1	2	1	1				4.3 ± 0.293	1.502 ± 0.202	1.439
X-2	200	52	0.2	0.311 ± 0.047	0.219	0.403	154	41	4	1												0.26 ± 0.036	1.014 ± 0.099	0.144
Y-2	200	121	0.9	0.806 ± 0.052	0.705	0.907	118	60	14	3	3	1		1								0.605 ± 0.055	1.593 ± 0.100	5.94
Z-2	200	352	2.2	2.463 ± 0.135	2.198	2.728	44	54	40	40	16	3	2	1								1.76 ± 0.094	1.161 ± 0.100	1.604

Dose estimates from two separate blind tests with 6 doses were used to evaluate the linear calibration curve covering 0-10 Gy. Four dose estimates were within the 95% CL and the other two (Y-1 and X-2) were just outside.

Table 3. Post-RT distribution of excess PCC objects in cancer patients undergoing radiotherapy.

Sample ID	Cells Scored	Aberrations	Distribution of Excess Objects																										Var/Mean±SE	u
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	…	22	…	27	…	30		
RTG-12(3)	67	86	27	19	9	4	4	3	1																				1.837 ± 0.173	4.836
RTG-13(3)	86	98	46	23	9	4			2								1					1								6.322 ± 0.153	34.87
RTG-14(3)	200	159	144	36	9	3	2		1	1					1			1			1								1	10.640 ± 0.100	96.42
RTG-24(3)	200	125	141	35	10	8	3	1						1							1									4.719 ± 0.100	37.24
RTG-25(3)	200	306	108	43	22	5	3	4	2	2	1	3	2			2			1						1		1			7.685 ± 0.100	66.79

Overdispersion was observed in all five samples indicating partial-body exposure.

Table 4. PCC dose estimates for pre-RT patient samples with DCA results used in comparison.

Patient Samples	Calyculin A-induced PCC						DCA					
	No. of Cells Scored	No. of PCC Objects	Yield ± SE	Dose Estimate (Gy) ± SE	Lower 95% CL	Upper 95% CL	No. of Cells Scored	No. of Dicentrics	Yield ± SE	Dose Estimate (Gy) ± SE	Lower 95% CL	Upper 95% CL
RTG-12(1)	94	27	0.287 ± 0.055	0.350 ± 0.069	0.216	0.484	500	0	0	0 ± 0.025	0	0.038
RTG-13(1)	200	47	0.235 ± 0.034	0.275 ± 0.045	0.186	0.364	500	0	0	0 ± 0.025	0	0.038
RTG-14(1)	200	37	0.185 ± 0.03	0.203 ± 0.042	0.121	0.286	500	4	0.008 ± 0.004	0.137 ± 0.066	0.007	0.266
RTG-24(1)	200	33	0.165 ± 0.029	0.175 ± 0.040	0.095	0.254	500	0	0	0 ± 0.025	0	0.038
RTG-25(1)	200	110	0.55 ± 0.052	0.727 ± 0.053	0.624	0.83	500	0	0	0 ± 0.025	0	0.038

PCC dose estimates for pre-RT patient samples were significantly ($p = 0.048$) higher than those generated using DCA.

Table 5. PCC dose estimates for post-RT patient samples with DCA results used in comparison.

Patient Samples	Calyculin A-induced PCC						DCA					
	No. of Cells Scored	No. of PCC Objects	Yield ± SE	Dose Estimate (Gy) ± SE	Lower 95% CL	Upper 95%CL	No. of Cells Scored	No. of Dicentrics	Yield ± SE	Dose Estimate (Gy) ± SE	Lower 95% CL	Upper 95% CL
RTG-12(3)	67	86	1.28 ± 0.138	1.779 ± 0.199	1.389	2.17	461	100	0.217 ± 0.022	1.505 ± 0.087	1.334	1.676
RTG-13(3)	86	98	1.14 ± 0.115	1.573 ± 0.166	1.248	1.898	343	100	0.292 ± 0.029	1.792 ± 0.097	1.601	1.982
RTG-14(3)	200	159	0.795 ± 0.063	1.078 ± 0.043	0.993	1.164	500	48	0.096 ± 0.014	0.909 ± 0.085	0.743	1.075
RTG-24(3)	200	125	0.625 ± 0.056	0.835 ± 0.051	0.734	0.935	500	62	0.124 ± 0.016	1.069 ± 0.085	0.903	1.236
RTG-25(3)	200	306	1.53 ± 0.087	2.133 ± 0.126	1.885	2.381	500	38	0.076 ± 0.012	0.781 ± 0.084	0.615	0.946

Dose estimates for post-RT patient samples generated using PCC were statistically ($p = 0.406$) comparable to the results obtained from DCA.

Sample ID	Tumour Type	Gender	Dose Received (Gy)	No. of Fractions	Chemo/Biological Treatment
RTG-12	Breast	Female	48	15	EC-P/Carboplatin, 8 cycles
RTG-13	Endometrium	Female	45	25	none
RTG-14	Bladder	Female	30	5	none
RTG-24	Head and Neck	Male	55	20	none
RTG-25	Breast	Female	40.05	15	none

Supplementary table for RTGene 2 patients.