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Chromosome analysis in childhood cancer survivors and their offspring—No evidence for radiotherapy-induced persistent genomic instability

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Abstract

Suggestions that the induction of genomic instability could play a role in radiation-induced carcinogenesis and heritable disease prompted the investigation of chromosome instability in relation to radiotherapy for childhood cancer. Chromosome analysis of peripheral blood lymphocytes at their first in vitro division was undertaken on 25 adult survivors of childhood cancer treated with radiation, 26 partners who acted as the non-irradiated control group and 43 offspring. A statistically significant increase in the frequency of dicentrics in the cancer survivor group compared with the partner control group was attributed to the residual effect of past radiation therapy. However, chromatid aberrations plus chromosome gaps, the aberrations most associated with persistent instability, were not increased. Therefore, there was no evidence that irradiation of the bone marrow had resulted in instability being transmitted to descendant cells. Frequencies of all aberration categories were significantly lower in the offspring group, compared to the partner group, apart from dicentrics for which the decrease did not reach statistical significance. The lower frequencies in the offspring provide no indication of transmissible instability being passed through the germline to the somatic cells of the offspring. Thus, in this study, genomic instability was not associated with radiotherapy in those who had received such treatment, nor was it found to be a transgenerational radiation effect.

Keywords

Chromosome aberrations; Genomic instability; Radiotherapy; Carcinogenesis

1. Introduction

In 1976, Nowell [1] proposed that genomic instability provides the driver for the process of carcinogenesis, this being characterised by the acquisition of increasing DNA sequence and chromosomal changes as part of the stepwise process of tumour progression [2,3]. The observation that radiation could result in persistent transmissible genomic instability has led to suggestions that this non-targeted effect, which results in de novo genetic changes arising in descendant cells, could play a role in radiation-induced carcinogenesis [4,5]. The phenomenon has been widely demonstrated following in vitro exposure for a range of endpoints and radiation conditions [6,7]. However, most reports of the induction of radiation-induced genomic instability in vitro use cells that are already cancer-prone, and it is notable that a recent study using normal fibroblasts failed to find any evidence of such an effect [8]. The mechanisms involved in radiation-induced transmissible genomic instability and their relationship with the direct induction of radiation-induced cellular damage remain to be elucidated, but one well-established marker is an increased frequency of chromatid aberrations in clonal descendants of irradiated cells many generations after exposure [7,9]. Chromatid aberrations, by their nature, are most likely to have arisen during the current cell division cycle and it has been suggested that the appearance of such de novo aberrations in descendants of irradiated cells may be related to apoptosis [10].

Studies of in vivo induction and transmission of persistent genomic instability are more limited and predominantly involve haemopoietic cells. Studies of mice irradiated in utero have provided conflicting results [11–13] although it is difficult to make comparisons because of differences in exposure regimes, cellular systems examined and cytogenetic endpoints. Cytogenetic studies suggesting that radiation-induced genomic instability could be transmitted in vivo in ablated mice transplanted with in vitro irradiated bone marrow [7,14] could not be confirmed [15] with this latter study also failing to find evidence of chromosomal instability in bone marrow cells following in vivo irradiation. Preliminary results from an on-going mouse study involving sequential sampling of polychromatic erythrocytes following whole-body irradiation have also failed to find an effect [16]. In contrast, in vivo induction and transmission of chromosomal instability has been demonstrated in mouse mammary tissue [17].

Few opportunities have arisen to study the in vivo induction and transmission of genomic instability in humans, and the studies that have been reported have demonstrated negative results. No suggestion of enhanced genomic instability was found when lymphocytes from a man with a 40-year history of internal exposure to alpha particles from thorium dioxide were examined for chromatid aberrations, although the presence of stable chromosomal aberrations provided evidence of ongoing bone marrow exposure [18]. Follow-up studies of men accidentally exposed to a cesium-137 source found no evidence of genomic instability in short-term lymphocyte cultures, although long-term cultures of both exposed and control individuals contained chromosomally unstable cells [19]. Furthermore, two studies in our laboratory involving sequential sampling of radiotherapy patients [20] and plutonium workers [21] found no evidence of increases in de novo chromatid aberrations in peripheral blood lymphocytes, although increased frequencies of stable chromosome aberrations indicated that viable descendants of irradiated bone marrow cells were present.

The suggestion that persistent genomic instability could be transmitted through the germline arose from a transgenic mouse study in which offspring of paternally irradiated mice exhibited increased mutations in the transgene in cells from the bone marrow [22]. Support for this came from the observation of increased chromosome damage, primarily gaps and chromatid breaks, in the bone marrow of the offspring of male mice injected with plutonium-239 [23]. This has subsequently led to a speculation that the induction of genomic instability in the germline is the mechanism whereby radiation exposure can result in heritable mutations in DNA

minisatellite regions [24]. Moreover, the instability can then be passed on through several generations resulting in persistently raised mutation rates [25]. Transgenerational genomic instability has also been suggested [26] as a possible mechanism to explain the reported elevated incidence of leukaemia [27] and stillbirths [28] in offspring of occupationally exposed radiation workers from the Sellafield nuclear reprocessing plant.

With advances in therapy, 75% of children diagnosed with childhood cancer can now expect to be long-term survivors and go on to lead relatively normal lives [29]. Many such individuals experience radiotherapy as part of their treatment and this leaves a potential legacy of increased risks for further malignancies and, where gonadal exposure has occurred, for heritable disease in subsequent offspring. A suggestion that one mechanism for these effects could be through the induction of persistent genomic instability [30] has led us to investigate the presence of this phenomenon in a group of childhood cancer survivors and their offspring. The survivors' partners provided a control group. Peripheral blood lymphocyte cultures from the survivors were analysed for chromosome aberrations since increased frequencies, particularly of chromatid aberrations, would be indicative of genomic instability being induced in the bone marrow precursor cells. Similarly, lymphocyte cultures from their offspring were examined for evidence of the inheritance of a generalised instability resulting from gonadal exposure.

2. Methods

2.1. Study group

Twenty-eight childhood cancer survivors who had received radiotherapy were identified from the Danish Childhood Cancer Survivor Cohort which comprises 4676 survivors notified to the Danish Cancer Registry with cancer at age <20 years between 1943 and 1996 who survived until onset of fertility (age 15 years) [31]. Survivors had to be alive on or born after April 1, 1968, when the national Central Population Register (CPR) was established and a unique personal identification number was assigned for all citizens. A search in the CPR resulted in identification of offspring and partners. Blood samples were taken from these 28 survivors and their families as a pilot study for the investigation of a range of genetic endpoints associated with germ cell mutagenesis and cancer susceptibility [31]. Information on cancer in relatives, smoking habits and medication was obtained from a short questionnaire completed by the parents. Approval for the study was obtained from the Danish Scientific Ethical Committee and the Danish Data Protection Agency. Blood samples were taken and transported by courier to the UK in 10 shipments over 13 months. The samples were hand-inspected at customs and a piece of dental film was included with each shipment to ensure that they had not been exposed to X-rays. Blood samples within each family were coded at the time of blood draw to avoid identification of cancer survivor, partner and offspring and were further coded prior to cytogenetic analysis so that families could not be identified. Samples were successfully taken from all 28 cancer survivors and their partners and 44 offspring. For one family it was not possible to obtain a blood sample from the offspring but these parents were still included in the study group (survivor T11). Analysis completed for four stable minisatellite loci confirmed biological paternity and maternity for all offspring.

2.2. Dosimetry

Data on individual cancer types and radiotherapy treatment are shown in Table 1. Organ doses for individual patients were reconstructed based on the information available in radiotherapy records. The records were submitted to The University of Texas, M.D. Anderson Cancer Center, Houston, Texas, for data abstraction and dose modelling [31,32]. Doses are provided for testes, ovary (both minimum and maximum doses) and bone marrow (weighed average bone marrow dose and maximum dose to any part of the bone marrow). Two of the childhood cancer survivors (T04 and T24) have had further malignancies. In the case of T04, this occurred

before conception and both bone marrow and ovarian doses administered as treatment for the second cancer are included. However, diagnosis and treatment for the second malignancy in T24 occurred several years after the birth of her children and therefore, whilst the bone marrow dose associated with treatment for the second cancer is included, the ovary dose is not relevant for this study and has been excluded.

2.3. Cell culture and chromosome analysis

Peripheral blood lymphocytes were cultured at 37 °C in RPMI 1640 medium supplemented with 15% foetal bovine serum and 2% phytohaemagglutinin. Colcemid was added for the final 4 h of culture at a final concentration of 0.1 g/ml. Bromodeoxyuridine (BrdU) was added to all the cultures to enable the selection of first division metaphases for analysis. Initial samples were cultured for 48 h but this resulted in a high proportion of second division cells, and so, culture time was reduced to 46 h for later samples. Harvesting was performed according to standard techniques with exposure to 75 mM potassium chloride and fixation with 3:1 methanol:acetic acid solution. Slides were prepared following storage of the fixed cells at -20 °C for at least 24 h and stained according to the fluorescence plus giemsa method.

All analyses were carried out on coded slides by one person who was unaware of the nature of the study. Between 200 and 500 cells were analysed for each individual. For six individuals it was not possible to obtain a minimum of 200 cells for analysis and they were removed from the study, leaving a final population of 25 survivors (T23, T27 and T28 excluded), 26 partners (those of T20 and T23 excluded) and 43 offspring (child of T05 excluded) (Table 2). All observable aberrations in apparently complete cells containing 46 centromeres and with all chromosome material present were included in the analysis. These included unstable chromosome aberrations, i.e. dicentrics, centric rings and excess fragments, chromatid aberrations, i.e. gaps, breaks and exchanges, and chromosome gaps. Aberration frequencies were compared using a χ^2 test (with one degree of freedom) at the 95% significance level.

3. Results

Data on age, smoking, aberration frequencies and aberrant cell frequencies are shown in Table 2 for the three study groups. The two adult groups are of similar age and contain similar numbers of current smokers. Comparison of the frequencies of dicentrics showed a statistically significant increase in aberrations in the survivor group when compared to that of the partner group ($\chi^2_1=22.51$, $P < 0.001$) or the offspring group ($\chi^2_1=44.57$, $P < 0.001$). Although a higher frequency of dicentrics was found in the partner group compared with the offspring group, this did not reach statistical significance ($\chi^2_1=0.66$, $P = 0.416$). The survivor and partner groups had similar frequencies of acentrics, but the frequencies in both groups were significantly raised in comparison with the offspring group (survivors versus offspring $\chi^2_1=17.69$, $P < 0.001$; partners versus offspring $\chi^2_1=9.57$, $P = 0.002$). Unstable cells containing any chromosome-type aberration, i.e. dicentrics, centric rings, and excess acentric fragments, were significantly raised in the survivor group when compared to the partner group ($\chi^2_1=4.02$, $P = 0.045$) or offspring ($\chi^2_1=27.64$, $P < 0.001$). A significant difference was also observed for unstable cell frequencies between the partner group and offspring group ($\chi^2_1=8.52$, $P = 0.004$).

The frequencies of chromatid aberrations plus chromosome gaps were similar for the partner and survivor groups, but the offspring group had a significantly lower frequency in comparison with both adult groups (survivors versus offspring $\chi^2_1=4.22$, $P = 0.04$; partners versus offspring $\chi^2_1=5.53$, $P = 0.019$). Similar results were found when frequencies of cells with chromatid aberrations and chromosome gaps were compared (survivors versus partners not significant;

survivors versus offspring $\chi^2_1=4.39$, $P = 0.036$; partners versus offspring $\chi^2_1=4.16$, $P = 0.041$). Two survivors (T06 and T18) received negligible bone marrow irradiation, but removal of these cases from the analysis results in a frequency of $4.12 \pm 0.63 \times 10^{-3}$ for cells with chromatid aberrations plus chromosome gaps which is similar to the value of $3.95 \pm 0.60 \times 10^{-3}$ for the whole survivor group.

Since it was not possible to obtain an age-matched control group for the offspring group, comparisons were made between those with negligible parental gonadal exposure and the remainder (Table 3). Six individuals in the survivor group had received negligible gonadal irradiation of ≤ 0.05 Gy, although one of these (T11) had no offspring. The frequency of cells with chromatid aberrations plus chromosome gaps for the 10 offspring of the remaining five (T06, T13, T18, T19, and T24) is not statistically different from the frequency obtained for the 33 offspring with parental gonadal exposure > 0.05 Gy (Table 3). Similarly, no differences were seen between the two groups for any of the other aberration categories.

4. Discussion

Chromosome analysis of peripheral blood lymphocytes shortly after radiation exposure is a well-established technique for dose estimation in cases of accidental over-exposure [33]. Using a standard block staining technique to examine cells in their first in vitro metaphase, it is possible to identify a range of asymmetrical chromosome-type aberrations, e.g. dicentrics, rings, acentric fragments, resulting from G0 irradiation in vivo. However, cells carrying such aberrations are unstable and are unable to go through repeated division cycles. Studies of radiotherapy patients have indicated that, whereas treatment regimes that result in some peripheral blood exposure will yield high frequencies of unstable cells, these decline rapidly with time [20]. Nevertheless, examination of the distribution of the 31 dicentrics in the 11,138 cells from the survivors revealed a significant deviation from Poisson expectations ($P < 0.001$), indicating that residual chromosome damage still remains from the localised radiation treatments. This is reflected in the dicentric frequency which is significantly raised in comparison with the partner group of comparable age and the offspring group. Studies involving a wide age range have generally shown that frequencies of dicentrics increase with age [34–37] and, although the difference between the partner group and the offspring group did not reach statistical significance, there is a suggestion of a similar effect in the present study. Studies that have examined acentric frequencies in relation to age have reported both increases [36,37] and no effect [34], with the present study revealing a significant increase in acentrics in the partner group compared with the offspring group. Previous studies have reported no age effect for frequencies of chromatid aberrations [34,37]. However, in the present study, chromatid aberrations were significantly raised in the partner group compared with the offspring group. To ensure that any suggested age-attributable differences between the partner group and the offspring were not masking a parental gonadal irradiation effect, further comparisons were made between the offspring of those with gonadal doses of ≤ 0.05 Gy and the remainder (Table 3). No significant differences were observed.

Frequencies of chromatid-type aberrations are not expected to be raised following irradiation of G0 lymphocytes, but have been reported as the aberrations most commonly seen at increased frequencies in descendants of cells that, although irradiated, remained viable [7,9]. These increases in de novo aberrations are thought to be indicative of genomic instability induced in the irradiated cell and transmitted to its progeny. Comparison of survivor and partner groups provides no evidence for this effect in the present study, despite treatment regimes that resulted in a wide range of bone marrow doses (Table 1).

In a previous study, we found no evidence for the induction of persistent or late-manifesting genomic instability in sequential samples from adult patients who had received radiotherapy

for a range of malignant conditions [20]. It was subsequently suggested that the failure to observe such an effect could be due to a lack of significant bone marrow irradiation and to the small number of cells analysed per patient [16]. In reporting this earlier work, we neglected to mention that G-banding analysis results obtained several years after exposure from all cases but one indicated persistent and significantly raised frequencies of translocations, suggesting that the bone marrow irradiation was not inconsequential. Some of these cases have been the subject of a subsequent report [38]. A further study of radiation workers with internal deposits of plutonium and raised frequencies of translocations in peripheral blood lymphocytes also failed to detect any evidence of the induction of persistent chromosomal instability [21]. Cells carrying translocations are stable and the presence of increased frequencies of translocations in peripheral blood lymphocytes many years after exposure is thought to reflect stem cell irradiation. Thus, if descendants of irradiated cells are present in peripheral blood lymphocytes, any genomic instability, as measured by increased chromatid-type aberrations plus chromosome gaps, should have been detected. In the present study, analysis for stable chromosome aberrations has not been undertaken, since bone marrow doses have been computed for each individual and provide evidence of irradiation of haemopoietic precursor cells.

The present study also provided a unique opportunity to investigate the possibility that genomic instability could be transmitted through the germline and observed in somatic cells of the offspring of irradiated parents. However, no evidence for a transgenerational effect was found since neither chromosome nor chromatid aberration frequencies were raised in the offspring in comparison with the partner control group, but rather were decreased, probably reflecting the difference in age between the two groups. This lack of effect in the offspring was confirmed, when in order to control for any influence of age, comparisons were made between the frequencies in the offspring of the five survivors with ≤ 0.05 Gy gonadal dose and the remainder.

The recognition that second cancers in patients who have received radiotherapy are related to the treatment for their first malignancy provided the focus for a recent review which examined whether such cancers could have arisen from radiation-induced genomic instability [39]. However, it was concluded that current studies do not provide sufficient evidence to determine if the phenomenon contributes to secondary malignancies after radiotherapy. Moreover, the search for such a mechanism for radiation-induced carcinogenesis may be misguided in light of a recent challenge to the prevailing view that genomic instability is a requirement for the carcinogenic process [40]. Rather, in most cases, tumour growth is driven by selection and there is no convincing evidence that tumour initiation or progression is generally driven by genomic instability. Since the present study provides no evidence that in vivo radiation exposure induces persistent genomic instability, the findings contribute to increasing doubt that such an effect provides a potential mechanism for possible somatic and heritable effects in those exposed to radiation.

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Cancer type and radiation dosimetry for 28 Danish childhood cancer survivors—total dose to bone marrow and preconception dose to gonads

Table 1

Patient ID	Gender	Primary disease	Body site treated	Age at time of treatment (years)	Age at time of sampling (years)	Testes dose (Gy)	Ovary min/max dose (Gy)	Total active bone marrow — weighted average (Gy)	Total active bone marrow max dose (Gy)
T01	F	Hodgkin's disease	Chest (mantle) + spleen	15	35	—	0.28/0.28	14.00	37.00
T02	F	Hodgkin's disease	Chest (mantle)	11	34	—	0.11/0.11	9.00	37.00
T03	M	Rhabdomyosarcoma	Para-aortic + groin	9	34	0.25	—	6.90	30.00
T04 ^a	F	Hodgkin's disease	Chest (mantle)	15	36	—	0.31/0.31	9.20	37.00
		Thyroid cancer	I ¹³¹	30					
T05	M	Hodgkin's disease	Chest (mantle) + abdomen/pelvis (inverted Y)	10	36	1.20	—	16.00	37.00
T06	F	Teratoma	Mediastinum	0.1	33	—	<0.01/<0.01	0.01	0.15
T07	F	Hodgkin's disease	Chest (mantle)	19	29	—	0.08/0.08	10.00	38.00
T08	M	Neuroblastoma	Spine (T11-L3)	0.6	32	0.21	—	2.40	30.00
T09	M	Wilms' tumor	Kidney	7	33	0.17	—	5.50	30.00
T10	F	Wilms' tumor	Kidney	4	25	—	0.66/0.69	3.70	30.00
T11	M	Non-Hodgkin's lymphoma	Neck + tonsil	8	30	0.03	—	2.30	39.00
T12	F	Hodgkin's disease	Chest (mantle)	14	25	—	0.10/0.10	10.00	37.00
T13	F	Lymphoepithelioma	Rhinopharynx + neck	20	30	—	0.05/0.05	5.00	60.00
T14 ^b	M	Ewing's sarcoma	Tibia	7	43	0.30	—	0.50	38.00
T15	M	Pneocytoma	Brain + spine	19	35	0.23	—	18.50	54.40
T16	F	Hodgkin's disease	Chest (mantle) + spleen	20	33	—	0.29/0.29	15.00	37.00
T17	M	Germinoma	Brain + spine	17	24	0.17	—	13.60	51.20
T18	M	Malignant schwannoma	Forearm (electron treatment)	19	35	<0.01	—	0.01	30.00
T19	M	Hodgkin's disease	Chest (mantle)	17	35	0.04	—	11.00	37.00
T20	F	Hodgkin's disease	Chest (mantle) + spleen	17	33	—	0.29/0.29	16.00	37.00
T21	F	Hodgkin's disease	Chest (mantle)	19	35	—	0.09/0.09	11.00	35.00
T22	M	Wilms' tumor	Kidney	1	31	0.21	—	3.70	35.00
T23	M	Wilms' tumor	Kidney	5	32	0.20	—	5.30	39.00

Patient ID	Gender	Primary disease	Body site treated	Age at time of treatment (years)	Age at time of sampling (years)	Testes dose (Gy)	Ovary min/max dose (Gy)	Total active bone marrow — weighted average (Gy)	Total active bone marrow max dose (Gy)
T24 ^c	F	Lymphoblastic lymphoma	Brain	14	36	—	0.01/0.01	7.90	50.00
		Breast cancer	Breast +pelvis	33					
T25	F	Neuroblastoma	Chest + abdomen + pelvis	1	36	—	9.20/9.20	4.80	21.00
T26	F	Hodgkin's disease	Chest (mantle)	19	37	—	0.08/0.08	10.00	37.00
T27	F	Wilms' tumor	Kidney	2	36	—	1.20/1.30	4.50	30.00
T28	F	Wilms' tumor	Kidney	2	34	—	1.60/1.70	4.60	32.00

^aOvary and bone marrow doses associated with treatment for both malignancies.

^bSubsequent diagnosis osteomyelitis.

^cOvary dose associated with treatment for first malignancy, bone marrow dose associated with treatment for both malignancies.

Table 2

Group data on aberration frequencies for childhood cancer survivors, partners and offspring

	Survivors	Partners	Offspring
Number of individuals	25	26	43
Mean age (years) (range)	33 (24–43)	34 (23–43)	5 (0.4–14)
Mean-weighted average bone marrow dose (Gy) (range)	8.24 (0.01–18.50)	–	–
Mean parental gonadal dose (Gy) (range)	–	–	0.64 (<0.01–9.20)
Number of current smokers	8	6	0
Number of cells analysed	11,138	11,829	20,854
Unstable chromosome aberrations			
Number of dicentrics (centric rings)	31 (2)	4 (1)	4 (0)
Number of excess acentric fragments	23	18	10
Frequency of dicentrics $\pm$ S.E. $\times 10^{-3}$	2.78 ± 0.50	0.34 ± 0.17	0.19 ± 0.10
Frequency of excess acentric fragments $\pm$ S.E. $\times 10^{-3}$	2.07 ± 0.43	1.52 ± 0.36	0.48 ± 0.15
Frequency of cells with dicentrics, centric rings and excess acentric fragments $\pm$ S.E. $\times 10^{-3}$	2.96 ± 0.52	1.69 ± 0.38	0.62 ± 0.17
Chromatid aberrations + chromosome gaps			
Number of chromatid aberrations + chromosome gaps	44 + 1	48 + 2	54 + 2
Frequency of chromatid aberrations+ chromosome gaps $\pm$ S.E. $\times 10^{-3}$	4.04 ± 0.60	4.23 ± 0.60	2.69 ± 0.36
Frequency of cells with only chromatid aberrations + chromosome gaps $\pm$ S.E. $\times 10^{-3}$	3.95 ± 0.60	3.89 ± 0.57	2.59 ± 0.35

Table 3

Additional data on offspring

	Offspring of survivors with gonadal dose ≤0.05 Gy	Offspring of survivors with gonadal dose >0.05 Gy
Number of individuals	10	33
Mean age (years) (range)	8 (0.4–14)	5 (0.6–14)
Mean parental gonadal dose (Gy) (range)	0.02 (<0.01–0.05)	0.78 (0.08–9.20)
Number of cells analysed	4632	16222
Unstable chromosome aberrations		
Number of dicentrics	0	4
Number of excess acentric fragments	1	9
Frequency of dicentrics ± S.E. × 10 ⁻³	0	0.25 ± 0.12
Frequency of excess acentric fragments ± S.E. × 10 ⁻³	0.22 ± 0.22	0.55 ± 0.18
Frequency of cells with dicentrics, centric rings and excess acentric fragments ± S.E. × 10 ⁻³	0.22 ± 0.22	0.74 ± 0.21
Chromatid aberrations + chromosome gaps		
Number of chromatid aberrations + chromosome gaps	14 + 1	40+1
Frequency of chromatid aberrations + chromosome gaps ± S.E. × 10 ⁻³	3.24 ± 0.84	2.53 ± 0.39
Frequency of cells with only chromatid aberrations + chromosome gaps ± S.E. × 10 ⁻³	3.24 ± 0.84	2.40 ± 0.38