

Original Research Article

Persistence of radiation-induced aberrations in patients after radiotherapy with C-ions and IMRT



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ABSTRACT

Background and purpose: Chromosomal aberrations in peripheral blood lymphocytes are a biomarker for radiation exposure and are associated with an increased risk for malignancies. To determine the long-term cytogenetic effect of radiotherapy, we analyzed the persistence of different aberration types up to 2.5 years after the treatment.

Materials and methods: Cytogenetic damage was analyzed in lymphocytes from 14 patients that had undergone C-ion boost + IMRT treatment for prostate cancer. Samples were taken immediately, 1 year and 2.5 years after therapy. Aberrations were scored using the multiplex fluorescence in situ hybridization technique and grouped according to their transmissibility to daughter cells.

Results: Dicentric chromosomes (non-transmissible) and translocations (transmissible) were induced with equal frequencies. In the follow-up period, the translocation yield remained unchanged while the yield of dicentrics decreased to $\approx 40\%$ of the initial value ($p = 0.011$ and $p = 0.001$ for 1 and 2.5 years after compared to end of therapy). In 2 patients clonal aberrations were observed; however they were also found in samples taken before therapy and thus were not radiotherapy induced.

Conclusion: The shift in the aberrations spectrum towards a higher fraction of translocations indicates the exposure of hematopoietic stem and progenitor cells underlining the importance of a careful sparing of bone marrow during radiotherapy to minimize the risk for secondary cancers.

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1. Introduction

Radiation exposure (accidental as well as therapeutic) is associated with an increased cancer risk [1–3]. External radiotherapy of deep-seated organs unavoidably delivers considerable doses to the surrounding tissue. Improved therapy outcomes and longer patient survival shift the focus to possible late effects, including the risk for secondary malignancies. Leukemia is of particular

importance, because the time interval between exposure and disease development is relatively short [4]. As epidemiological data for secondary cancers are not available for new radiotherapy techniques (e.g. dose escalation, hypofractionation, particle therapy), approaches for their prediction are needed. We suggest the ratio of dicentrics to translocations in peripheral blood lymphocytes (PBLs) of patients after therapy as a measure for the damage to hematopoietic stem and progenitor cells (HSPCs) and thus for leukemia risk.

Chromosomal aberrations in PBLs are a validated biomarker for cancer risk [5,6]. Radiation induces translocations (simple reciprocal exchanges between two chromosomes) and dicentrics (simple exchanges between two chromosomes resulting in one chromosome with two centromeres and one without centromere) with similar frequencies [7,8]. Translocations are transmissible to daughter cells; they can be found in individuals many years after radiation exposure [9] and are regarded as a biomarker for the

Abbreviations: BM, bone marrow; BrdU, Bromodeoxyuridine; FISH, Fluorescence in situ hybridization; FPG, fluorescence plus Giemsa staining; GTV, gross tumor volume; HSPCs, hematopoietic stem/progenitor cells; mFISH, multiplex fluorescence in situ hybridization; PBLs, peripheral blood lymphocytes.

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long-term risk for secondary malignancies [10]. Dicentric are non-transmissible, therefore their frequency decreases with cell proliferation. As PBLs are normally in G0-phase and do not undergo cell divisions, a change in the ratio of dicentric to translocations is attributable to the proliferation of HSPCs carrying transmissible aberrations (predominantly translocations) which produce aberrant progeny that replace the PBLs which are reaching the end of their natural lifespan. Therefore, the long-term persistence of translocations can be regarded as an indicator for aberrant HSPCs. However, after radiotherapy the translocation yield is not necessarily constant, several studies report a decline of the translocation yield with time [11,12], some even a return to control level [13]. The creation of multi-aberrant cells by the high doses applied in radiotherapy is discussed as the underlying mechanism. Subsequently translocations in cells also carrying non-transmissible aberrations will disappear [14].

To address the question of therapy-induced aberrations in PBLs induced by the novel approach of high-LET radiotherapy, we previously analyzed chromosomal aberrations in patients undergoing radiotherapy for prostate cancer using both IMRT and a C-ion + IMRT combination [15,16] and detected no cytogenetic signature of high-LET C-ions and no difference in the aberration yield and spectrum after therapy between C-ion boost + IMRT and IMRT alone. A 1-year follow-up for some patients by means of the mFISH (multiplex fluorescence in situ hybridization) technique revealed a shift in the aberration spectrum towards transmissible types, indicating the induction of aberrations in HSPCs. This motivated a longer observation period. During the radiotherapy of prostate cancer, the femur heads and hip bones receive a substantial dose both by C-ion radiotherapy and IMRT [17,18], thus induction of aberrations in HSPCs within these structures can be expected. To relate the observed aberration pattern to bone marrow irradiation, treatment plans were analyzed.

For completeness, the chromosome data were checked for the presence of clonal aberrations, because radiation exposure of HSPCs can give rise to clonal aberrations observable in PBLs. Clonal aberrations [19], were found, for example, in the blood of atomic bomb survivors [20], Chernobyl nuclear power plant accident victims and clean-up workers [21,22] and Thorotrast patients [23], but also in control individuals [22,24–26].

2. Materials and methods

2.1. Patients

Blood samples were obtained at the end of ($n = 14$), one year ($n = 13$) and 2–3 years ($n = 6$) after therapy from patients diagnosed with prostate cancer, which had undergone a combined radiotherapy with accelerated C-ions followed by IMRT. These patients represent one group from a previous cytogenetic study (for details see [15,16]). For practical reasons, only 6 blood samples from 5 patients (one patient donated two samples with a time interval of 6 months) could be collected 2–3 year after therapy and will be referred to as “2.5 years”. The study was approved by the local ethical committee and all patients signed an informed consent form.

The patients were treated with a scanned C-ion boost of 18 GyE applied to the gross tumor volume (GTV) in two opposing fields and 5–7-field-step-and-shoot IMRT delivering 60 Gy (median dose) to the planning target volume [27]. Treatment plans were calculated with TRiP98 [28] and KonRad [29], respectively. GTVs had similar sizes of $64.2 \pm 11.9 \text{ cm}^3$ that resulted in very similar dose distributions, particularly for the C-ion boost. For the C-ion treatment, active bone marrow (BM) irradiation was limited to the femoral heads, part of the acetabula, and, depending on exact patient geometry, a portion of the pubes immediately adjacent to the prostate. IMRT affected larger normal tissue volumes, covering the marrow of the entire lower pelvis with a highly inhomogeneous dose. One patient's dose distributions in two different axial planes for ion and photon treatment modality are presented in Fig. 1. For the IMRT treatment, the dose to the BM was determined by defining a contour enclosing the entire normal tissue in the irradiated lower pelvis volume for two patients (one for each field configuration). For the C-ion boost, the average dose to the femoral heads (for which contours were available) was found to be representative of the entire irradiated BM.

2.2. Collection, processing and staining of PBLs

Blood samples were drawn into Vacutainer CPT™ tubes (BD). Lymphocytes were isolated, stimulated to grow and cultured in

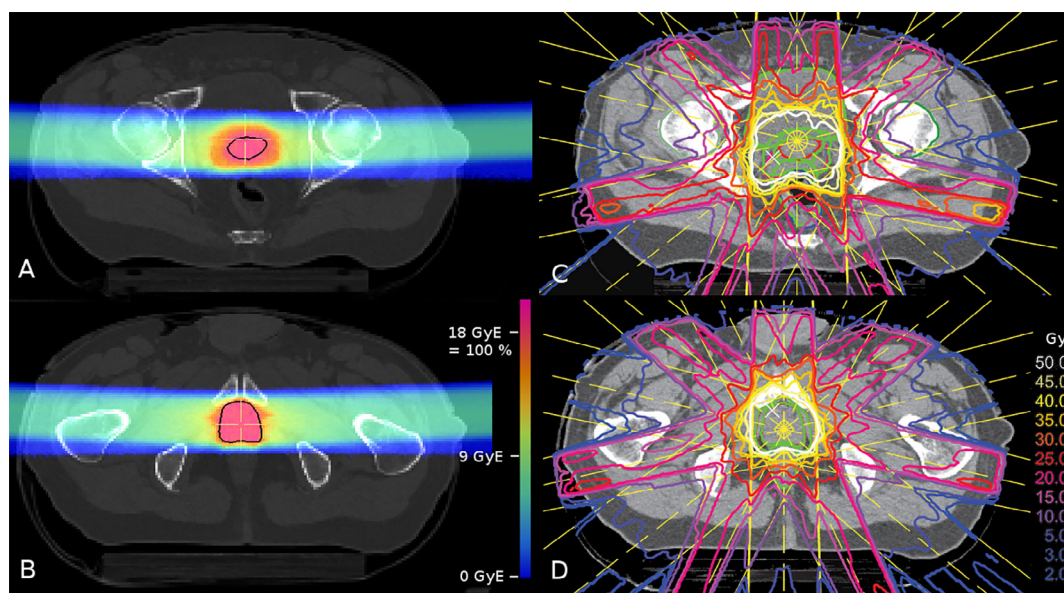


Fig. 1. Example slices of a treatment plan showing (A,B) the biologically effective dose applied by the C-ion boost and (C,D) the absorbed dose applied by IMRT (7 fields).

Table 1

Aberration types and ratios observed in PBLs of prostate cancer patients after treatment with C-ion + IMRT. Aberration yields are given per 100 cells (value $\pm$ error based on Poisson statistics), absolute numbers are given in parentheses.

Timepoint [years after therapy]	0	1	2.5
Number of samples	14	13	6
Number of analyzed metaphases	1658	1641	1207
Terminal deletions	3.6 ± 0.5 (60)	2.4 ± 0.4 (40)	2.2 ± 0.4 (26)
Translocations	6.6 ± 0.6 (109) ¹	8.0 ± 0.7 (132)	7.3 ± 0.8 (88)
Dicentrics	6.3 ± 0.6 (105)	4.1 ± 0.5 (67)	3.0 ± 0.5 (36)
Centric rings	0.42 ± 0.16 (7)	0.18 ± 0.11 (3)	0.25 ± 0.14 (3)
Inversions	0 (0)	0.12 ± 0.09 (2)	0.08 ± 0.08 (1)
Transmissible complex aberrations	0.36 ± 0.15 (6)	0.49 ± 0.17 (8)	0.58 ± 0.22 (7)
Non-transmissible complex aberrations	0.66 ± 0.20 (11)	0.79 ± 0.22 (13)	0.83 ± 0.26 (10)
Ratio dicentrics/translocation	0.95 ± 0.14	0.51 ± 0.15	0.41 ± 0.20
Ratio non-transmissible/transmissible complex aberrations	1.8 ± 0.5	1.6 ± 0.5	1.4 ± 0.5

¹ This includes 1 incomplete translocation according to [31].

the presence of 15 $\mu\text{g/ml}$ 5-bromo-2-deoxyuridine (BrdU) and metaphases were harvested at 48 h preceded by a 3 h treatment with colcemid (0.2 $\mu\text{g/ml}$). Chromosome samples were prepared according to standard protocols as described in [15].

One or two slides per sample were stained with the Fluorescence-plus-Giemsa (FPG) technique [30] to determine the proportion of cells in the first or a higher cell generation. For mFISH analysis additional slides were hybridized with the 24Xcyte mFISH probe (Metasystems, Germany) according to the manufacturer's protocol and examined using a Zeiss Axiolmager Z1 microscope. Automated metaphase search and image acquisition was performed with the Metafer4 software, and for image processing and analysis the Isis/mFISH software was used (both from Metasystems). If samples had less than 90% first cycle cells, differential staining was performed for the mFISH hybridized slides and only first cycle metaphases were analyzed [15].

Additionally, samples from four patients harboring a potentially clonal translocation were examined by 2-color FISH specific for the chromosomes in question (Metasystems). At least 100 metaphases were analyzed per mFISH sample, while for 2-color FISH, more than 1000 metaphases per sample were scored.

To relate changes in the aberration spectrum of patients' PBLs to the turnover of cells, an *in vitro* study was performed. PBLs from one healthy donor (non-smoker) were isolated, resuspended in medium and irradiated with X-rays (250 kV, 20 mA, 1 mm Cu + 1 mm Al filtering). Cells were cultivated for 72 h in the presence of BrdU. Then, chromosome spreads were prepared and mFISH in combination with differential staining was performed to identify cells in first, second and third mitosis as described elsewhere [15].

2.3. Classification of aberrations and statistical analysis

All types of chromosome aberrations were recorded using mPAINT descriptors [31], namely excess acentric fragments not associated with an exchange, simple exchanges resulting from two breakpoints comprising translocations, dicentrics, ring chromosomes and pericentric inversions and complex exchanges originating from three or more breakpoints in at least two chromosomes. Subsequently, aberrations were grouped according to their transmissibility to daughter cells. Non-transmissible aberrations comprise excess acentric fragments, dicentric chromosomes as well as ring chromosomes, while translocations and inversions are transmissible. Likewise, complex exchanges were classified as non-transmissible if they contained an excess fragment, a dicentric or a ring chromosome or as transmissible if they harbored neither of those. Clonal aberrations were defined as at least three cells carrying identical aberrations [19]. Errors on the frequencies of aberrant cells and different types of aberrations were calculated using Poisson statistics. To determine statistical

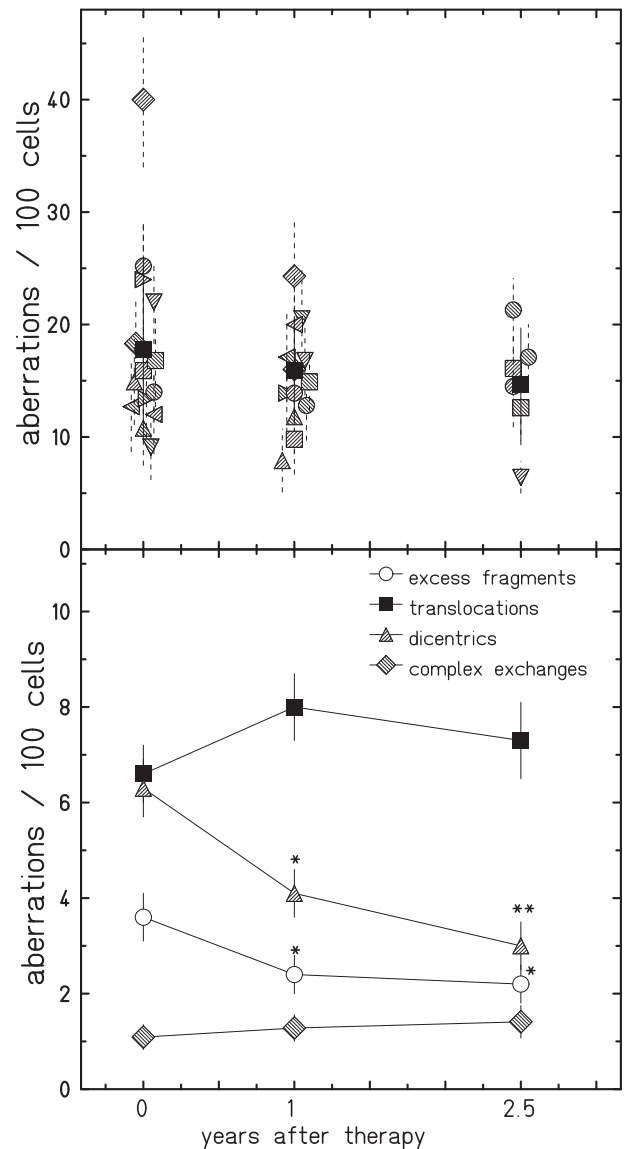


Fig. 2. Aberrations found in PBLs taken directly ($n=14$), 1 year ($n=13$) and 2.5 years ($n=6$) after therapy from prostate cancer patients that underwent a combined treatment with C-ions and IMRT. Upper panel: Aberration frequencies (patterned symbols) observed in individual patients in the follow-up period. Error bars are based on Poisson statistics. The solid symbols represent mean values $\pm$ standard deviation. Lower panel: Persistence of different aberrations types after radiotherapy. A significant (*, $p < 0.05$) or highly significant (**, $p < 0.01$) decrease relative to the value at the end of therapy is found for dicentrics and excess acentric fragments.

significance for the difference in aberration yields, a one-sided *t*-test was used.

3. Results

All aberrations detectable with mFISH were scored (see Table 1), and the total aberration yields are shown in the upper panel of Fig. 2. In agreement with previously published results [15], a slight but not statistically significant decrease in the aberration yield 1 and 2.5 years after therapy compared to the end of therapy was observed ($p = 0.24$ and $p = 0.20$, respectively).

As illustrated in Table 1, the majority of aberrations were simple exchanges, which are mainly comprised of translocations and dicentric chromosomes. The lower panel of Fig. 2 shows the time-course of the yield of the most frequent aberration types. Translocations and dicentrics were induced with equal frequencies. The translocation yield persisted over the entire observation period of 2.5 years, while the yield of dicentrics decreased significantly with time ($p = 0.011$ and $p = 0.001$ for 1 and 2.5 years after compared to the end of therapy). Excess acentric fragments also showed a statistically significant decrease in the follow-up period ($p = 0.047$ and $p = 0.042$). Complex chromosomal aberrations were divided into transmissible and non-transmissible forms, but no statistically significant changes were observed due to their low number and subsequently large error bars (see Table 1).

To relate the *in vivo* observed shift in the ratio of dicentrics to translocations to the turnover of cells, PBLs of a healthy donor were exposed *in vitro* to doses of 1–3 Gy X-rays, stimulated to grow and chromosome aberrations were analyzed 72 h later. Differential staining allowed discriminating first, second and third/higher mitoses after irradiation. The ratio of dicentrics to translocations is close to 1 *in vitro* in the first mitosis after irradiation as well as *in vivo* at the end of therapy (see Fig. 3). It declines to approximately 50% in second cycle and to approximately 25% in third cycle mitoses after irradiation. *In vivo*, in the samples from patients undergoing radiotherapy, the ratio of dicentrics to translocations decreases from close to 1 to about 0.5 in the first year after therapy and to about 0.4 within 2.5 years after therapy.

Data collected at 1 and 2.5 years after therapy were searched for the presence of clonal aberrations. No clonal aberration was found

according to the definition of three identical aberrations [19]. However, in 4 patients, we observed 2 metaphases with identical aberrations (in all cases translocations) which we considered “potentially clonal”. To confirm or reject these aberrations as clonal, we applied the time- and cost-effective 2-color FISH technique. In two of the 4 patients, the 2-color FISH analysis of more than 2000 metaphases did not show any additional copies of the aberration in question. In the other two patients, further copies of the potentially clonal aberration were found, confirming them as clonal aberrations. Fig. 4 shows examples of the clonal aberrations observed with mFISH, together with two examples of the 2-color FISH stained clones. The clonal aberrations were reciprocal translocations (1'-6)(6'-1) and (8'-14)(14'-8). Radiation-induced clonal aberrations need time to develop, as the aberrant HSPC undergoes many divisions producing aberrant progeny maturing into PBLs. To clarify whether the observed clonal aberrations were therapy-related, samples prepared before therapy and immediately after therapy were analyzed. These analyses revealed that the clonal aberrations were present before therapy in both patients and that their frequency increased with time (Table 2).

Clonal aberrations (especially large clones) can distort the results of the biological dosimetry. To check whether the two copies of the clonal aberration in the mFISH dataset influenced the results significantly, we compared the original results to the aberration yield if the clonal aberration is counted only once. This changes the observed aberration yield in patient A from 12.6 ± 3.3 to 11.8 ± 3.1 and the aberration yield in patient B from 13.9 ± 2.8 to 13.3 ± 2.8 . Thus, in the present study the clonal aberrations did not influence the results significantly.

As the shift in the aberration spectrum towards transmissible aberrations indicated a radiation-induced damage in the HSPCs, the dose to the bone marrow was investigated. On average, 240.3 cm^3 of pelvic BM (75% of which are considered active marrow [32]) were irradiated with a physical dose of $1.34 \pm 0.54 \text{ Gy}$ (mean $\pm$ SD) during the C-ion boost. The average BM dose corresponding to photon IMRT was 11.1 Gy with a standard deviation within the contoured volume of 11.5 Gy, reflecting the presence of extended volumes receiving either high or very low doses. The BM volume affected by this dose, including the lower pelvis and femoral heads and necks, amounts to approximately 14% of the

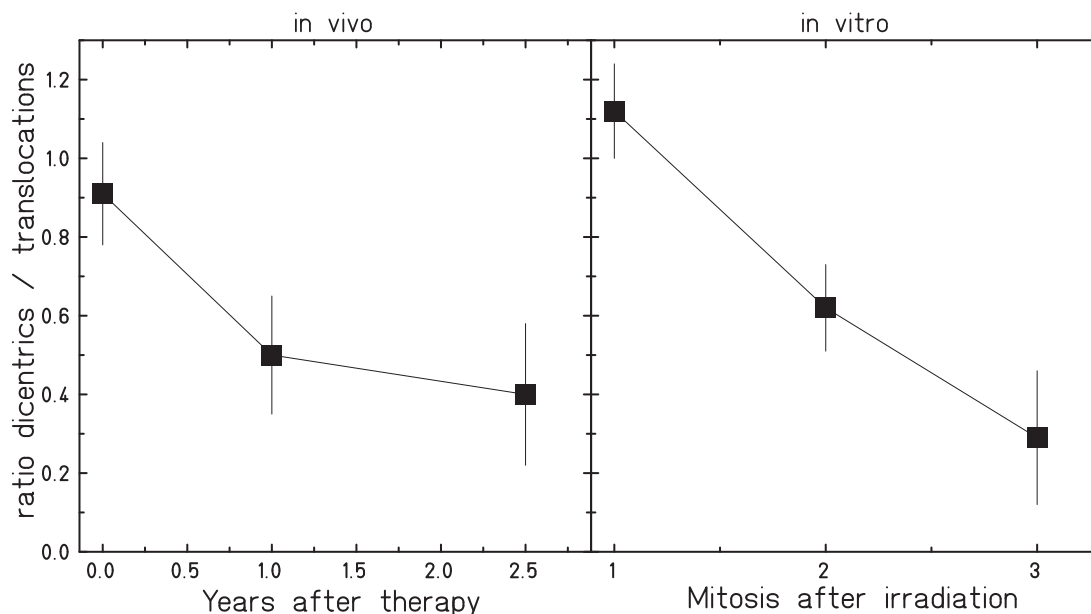


Fig. 3. Ratio of dicentrics to translocations. The left panel shows *in vivo* data from patients directly as well as 1 and 2.5 years after therapy. The right panel shows the ratio in the first, second and third/higher mitosis after *in vitro* X-ray irradiation.

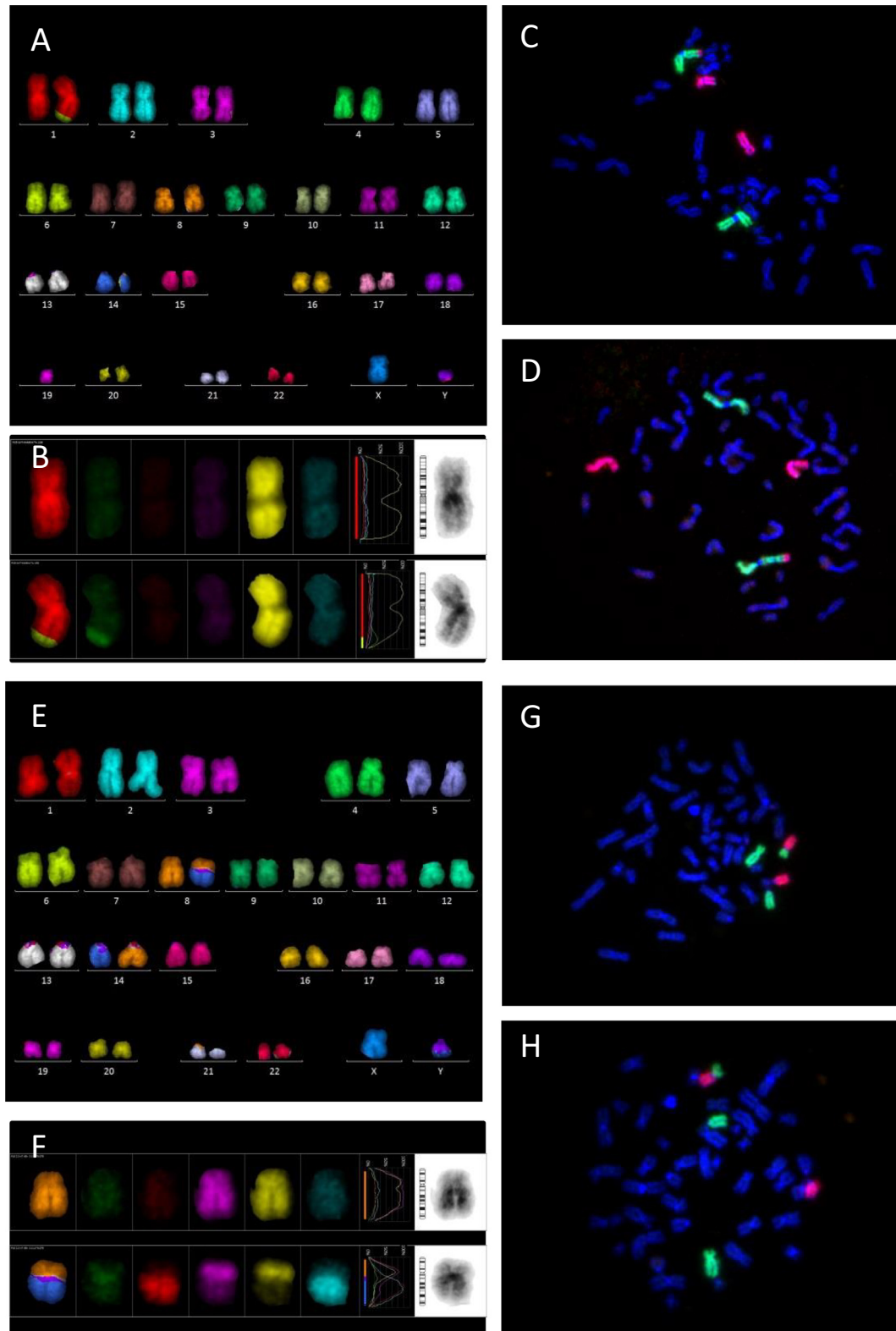


Fig. 4. PBLs from 13 prostate cancer patients that underwent radiotherapy were investigated for clonal aberrations. In 4 patients, “potentially clonal” aberrations (2 copies of an aberration) were found by mFISH; in 2 of these patients (referred to as patient A and patient B) the “potentially clonal” aberrations were confirmed as true clonal aberrations by application of 2-color FISH (see Table 2). **Fig. 4 A-D:** Reciprocal translocation (1'-6)(6'-1) found in patient A, stained with mFISH (Fig. A and B) and 2-color FISH (Fig. C and D). **Fig. 4 E-H:** reciprocal translocation (8'-14)(14'-8) found in patient B stained with mFISH (Fig. E and F) and 2-color FISH (Fig. G and H). For mFISH, in addition to the karyogram (Fig. A and E) the single color galleries (Fig. B and F) for a normal and aberrant chromosome are shown. In the single color galleries, translocations between chromosomes are visible as changes in the fluorescent color profiles along the chromosome.

Table 2

Number of analyzed metaphases and of cells carrying a clonal aberration observed in two patients (A and B) before and after therapy using 2-color FISH.

Patient	Sample	Analyzed cells	Clonal cells	Clonal cells per 1000
A	Before therapy	2053	3	1.5 ± 0.8
	End of therapy	–	–	–
	1 y. after therapy	1957	3	1.5 ± 0.9
	2.5 y. after therapy	1006	5	5.0 ± 2.2
B	Before therapy	1968	5	2.5 ± 1.1
	End of therapy	2602	7	2.7 ± 1.0
	1 y. after therapy	1980	5	2.5 ± 1.1
	2.5 y. after therapy	2861	13	4.5 ± 1.3

total active bone marrow [32] in adult age. Combining both treatment modalities, around 14% of the active bone marrow was exposed to 11.9 Gy (average).

4. Discussion

We analyzed chromosome aberrations in prostate cancer patients immediately as well as 1 and 2.5 years after radiotherapy. Application of mFISH technique allowed detecting all types of chromosomal interchanges and dividing them into transmissible and non-transmissible forms. The most frequent aberration types were (transmissible) translocations and (non-transmissible) dicentrics. The frequency of translocations remained constant up to 2.5 years after therapy, while the yield of dicentrics decreased with time after therapy. Both aberration types were induced with similar frequencies, but 2.5 years after therapy only 0.4 dicentrics per translocation remained.

Without any contribution of aberrant BM, the aberration yield in PBLs should decline with time, as the originally irradiated PBLs die and are replaced by new (undamaged) cells. Thus, a decrease in the aberration yield, but no change of aberration spectrum would be expected after therapy. We attribute the observed shift in the aberration spectrum (specifically in the ratio of dicentrics to translocations) to the radiation exposure of HSPCs. Radiation-induced transmissible aberrations in HSPCs are passed on to daughter cells which eventually enter the PBL-pool after maturation, while the yield of non-transmissible aberrations declines with time when the originally exposed lymphocytes reach the end of their natural lifespan. Wide ranges are discussed in literature for the lifespan of different PBL populations, e.g. 22 weeks and 3.5 years for different T cell subsets according to IAEA [14]. The radiation exposure of HSPCs in the BM was confirmed by analysis of the treatment plans and can explain the observed evolution of PBLs with transmissible aberrations from aberrant stem cells. In contrast to previous studies [10–13,33] the availability of patient CTs and dose distributions allowed us to quantitatively assess both the corresponding affected BM volume and dose.

Several studies investigated the persistence of translocations and dicentrics in cancer patients after radiotherapy, and the persistence of translocations after radiotherapy varied considerably [11–13,33–35]. The results ranged from a return to the control level within 20 months in testicular seminoma patients [13], over a >50% reduction within 12 months in head and neck cancer patients [12] to an elevated level of transmissible aberrations 23 years after radiotherapy for cervical cancer [33]. Apparently, the long-term persistence of translocations after radiotherapy depends on the location of the tumor, the target volume and the volume of exposed bone marrow as well as on the natural lifespan of PBLs. As the translocation yield alone cannot answer whether the observed aberrant cells are originally exposed cells or daughter cells of exposed HSPCs, we suggest the use of the translocation frequency together with the ratio of dicentrics to translocations to gain insight into the long-term cytogenetic effect of different radiotherapy regimes and the exposure of HSPCs.

In the present study, aberrations in 4 patients were identified as “potentially clonal” (2 cells with identical aberrations) by mFISH staining; subsequent 2-color FISH analysis confirmed the presence of a clonal aberration in two patients. Notably, in both patients the clonal aberration was already present in the samples taken before therapy, proving that it was not induced by the radiotherapy. Moreover, the fraction of clonal cells increased with time in both patients. The fraction of clonal cells within the PBL population seems small (1.5 – 5 per 1000 cells in patient A), but considering that the total number of lymphocytes in humans is estimated to be in the order of 10^{11} cells [36], and assuming a homogeneous distribution of the clonal cells within the PBL pool, this corresponds to 1.5×10^8 to 5×10^8 PBLs with the clonal aberration. To reach this number, a massive cell expansion is necessary. Radiation-induced clones are therefore only detectable after a sufficiently long time.

The majority of clonal aberrations described in literature are found in radiation-exposed individuals [20–22]. However, there are also several studies describing the presence of clonal aberrations in individuals not previously exposed to radiation. As the clonal aberrations observed in the present study were not therapy-induced, we compared the frequency (2 clones in 13 individuals, i.e. 15%) to control populations reported in the literature. Lucas *et al.* investigated samples from 35 control individuals and found 7 clones, i.e. in 20% of the individuals (defining two identical aberrations as clonal) [26]. In a study with 27 healthy donors, 3 clones were observed i.e. in 11% of the individuals (defining three identical aberrations as clonal) [25]. Considering the different staining techniques and scoring criteria as well as varying number of analyzed cells, our observations are in good agreement with literature data for control individuals. In the present study, the clonal cells present in the mFISH dataset did not significantly change the results, but large clones have to be considered in biological dosimetry, as they can significantly influence the results.

In summary, we showed that the translocation yield induced in PBLs of prostate cancer patients treated with C-ion boost + IMRT remains constant for at least 2.5 years, while the yield of dicentric chromosomes drops significantly in the same time, shifting the aberration spectrum in the follow-up after radiotherapy towards translocations. We attributed this shift to the production of PBLs with transmissible aberrations by irradiated HSPCs. The cytogenetic evidence for aberrant HSPCs underlines the importance of a careful sparing of bone marrow during radiotherapy to minimize the risk for secondary cancers.

Conflict of interest statement

The authors declare no conflict of interest.

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