Individual Radiosensitivity Does not Correlate with Radiation-Induced Apoptosis in Lymphoblastoid Cell Lines or CD^{3+} Lymphocytes

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Background and Purpose: Spontaneous and radiation-induced apoptosis of lymphoblastoid cell lines (LCLs) derived from healthy donors, cancer patients and donors with radiosensitivity syndromes as well as CD^{3+} lymphocytes from patients with ≥ grade 3 late toxicity were investigated as a possible marker for the detection of individual radiosensitivity. These investigations are based on the hypothesis that hypersensitive patients have reduced levels of apoptosis after in vitro irradiation as a result of a defect in the signaling pathway.

Material and Methods: Epstein-Barr virus-(EBV-)transformed LCLs derived from five healthy donors, seven patients with heterozygous or homozygous genotype for ataxia-telangiectasia or Nijmegen breakage syndrome and five patients with ≥ grade 3 late toxicity (RTOG) were investigated. In addition, CD^{3+} lymphocytes from 21 healthy individuals and 18 cancer patients including five patients with a proven cellular hypersensitivity to radiation were analyzed. Cells were irradiated in vitro with a dose of 2 and 5 Gy and were incubated for 48 h. Apoptotic rates were measured by the TUNEL assay followed by customized image analysis.

Results: Four out of seven radiosensitivity syndrome patients were identified to have an increased cellular radiosensitivity as determined by reduced apoptotic rates after irradiation of their respective LCLs. Comparatively, only two of the five hypersensitive cancer patients were clearly identified by reduced apoptotic rates. Spontaneous apoptotic rates were very homogeneous among all 39 samples from controls and patients, while lymphocytes of all cancer patients showed significantly lower radiation-induced rates.

Conclusion: Only a subgroup of hypersensitive patients may be identified by reduction of radiation-induced apoptotic rate. It is concluded that the hypothesis according to which hypersensitive cells have reduced levels of apoptosis is only conditionally true. The authors suggest that this assay can be used in combination with additional tests evaluating DNA double-strand break repair, cell-cycle control and chromosomal aberrations for the evaluation for individual hypersensitivity.

Key Words: Radiation-induced apoptosis · Individual radiosensitivity · Hypersensitivity · LCLs · Lymphocytes

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Individuelle Radiosensibilität korreliert nicht mit strahleninduzierter Apoptose in lymphoblastoiden Zelllinien oder CD^{3+}-Lymphozyten

Hintergrund und Ziel: Spontane und strahleninduzierte Apoptose in lymphoblastoiden Zelllinien (LCL) von Gesunden, Krebspatienten und Spendern mit Strahlenempfindlichkeitssyndromen sowie in CD^{3+}-Lymphozyten von Patienten mit Spättoxizität ≥ Grad 3 wurden mit dem Ziel untersucht, Apoptose als einen Marker zur Detektion von Strahlenempfindlichkeit zu verwenden. Dies basiert auf der Hypothese, dass erhöht strahlenempfindliche Zellen auf In-vitro-Bestrahlung mit erniedrigten Apoptoseraten reagieren, was durch eine Störung in der Signaltransduktion bedingt ist.

Material und Methodik: Epstein-Barr-Virus-(EBV-)transformierte LCL von fünf gesunden Spendern, sieben Patienten mit heterozygotem oder homozygotem Genotyp für Ataxia teleangiectatica oder Nijmegen-Breakage-Syndrom und fünf Patienten mit Spättoxizität ≥ Grad 3 (RTOG) wurden untersucht. Zusätzlich wurden CD^{3+}-Lymphozyten von 21 Gesunden und 18 Krebspatienten einschließlich fünf Patienten mit nachgewiesener Strahlenempfindlichkeit untersucht. Die Zellen wurden in vitro mit 2 und 5 Gy bestrahlt und für 48 h inkubiert. Die Apoptoseraten wurden mit dem TUNEL-Assay und einem selbst entwickelten Bildanalysesystem gemessen.

Ergebnisse: Nur vier der sieben Patienten mit Strahlenempfindlichkeitssyndromen wiesen eine durch erniedrigte Apoptoseraten belegte zelluläre Strahlenempfindlichkeit auf. Entsprechend konnten nur zwei der fünf erhöht strahlenempfindlichen Krebspatienten durch erniedrigte Apoptoseraten identifiziert werden. Die spontanen Apoptoseraten der Lymphozyten aller 39 Patienten

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und Kontrollen waren in sich sehr ähnlich, während die Lymphozyten der Krebspatienten erniedrigte strahleninduzierte Apoptoseraten aufwiesen.

Schlussfolgerung: Nur eine Untergruppe der erhöht strahlenempfindlichen Patienten kann durch erniedrigte Apoptoseraten identifiziert werden. Die Hypothese, dass strahlenempfindliche Zellen mit reduzierten Apoptoseraten auf In-vitro-Bestrahlung reagieren, gilt nur teilweise. Für die Detektion von erhöhter Strahlenempfindlichkeit wäre eine Kombination mit anderen Tests, die die DNA-Doppelstrangbruch-Reparatur, Zellzykluskontrolle und chromosomale Aberrationen untersuchen, zu empfehlen.

Schlüsselwörter: Strahleninduzierte Apoptose · Individuelle Strahlenempfindlichkeit · Überempfindlichkeit · Lymphoblastoide Zelllinien · Lymphozyten

Introduction

To identify the relatively small proportion of patients with an elevated risk for major acute and late radiation toxicity would be an attractive accomplishment for the practice of radiation oncology. By adapting single dose, total dose and fractionation of a given radiation treatment schedule to fit individual radiosensitivity, one would expect to have reduced numbers of individuals with severe late effects in a given patient population without compromising the intended tumoricidal effect of radiotherapy [29]. Concurrently, in those individuals who are found to be rather insensitive, dose escalation trials with the aim to increase total dose may be considered, leading to higher tumor cure rates.

While the known radiosensitivity syndromes are relatively rare diseases, around 1–4% of patients will experience late complications due to their increased intrinsic radiosensitivity. Patients with radiosensitivity syndromes, like ataxia-telangiectasia (AT) [32], Nijmegen breakage syndrome (NBS) [34], bloom syndrome and mutations in hMre11 [33] and ligase IV [25], have a markedly increased cellular radiosensitivity. Common features of patients with these genomic instability syndromes or DNA repair disorders are short stature, immunodeficiency, predisposition to cancer, elevated radiosensitivity, and a chromosomal instability. If such patients need radiotherapy, dose has to be reduced by a factor of 2–3 [14]; otherwise radiation treatment with a conventional total dose may result in severe toxicity and death [12].

However, in addition to these hypersensitivity syndromes, a proportion of patients exist who also suffer from severe treatment-related complications. While most gene defects encoding radiosensitivity syndromes are presently known, it is not obvious which mechanisms lead to the hypersensitivity of patients. Low-penetrance genes, gene-environment interactions, or both are discussed [36]. Patients with hypersensitivity have to be identified before the mechanisms causing radiosensitivity can be investigated. Clearly, fractionation schedule, total dose, radiation quality, dose distribution and volume, medication and other factors can influence late complication rates. Additionally, the time to occurrence of late complications may occur with an exponential kinetic [16]. This implicates that late complications occur in radiosensitive and "normal" individuals, but there is a higher probability for the sensitive group to experience severe complications. Therefore, only a subgroup of patients with severe late complications has a genetic predisposition to hypersensitivity against radiation.

Cells from patients with radiosensitivity syndromes can be identified by a two- to threefold increase in chromosomal aberrations as detected by fluorescence in situ hybridization (FISH) or cell inactivation by the clonogenic assay or other functional assays [18, 19, 24]. It is more difficult to evaluate and characterize patients that have moderately elevated radiosensitivity as defined by the normal distribution of radiotherapy patients. To accomplish this in vitro, functional assays are used for testing DNA repair, misrepair, cell-cycle control and cell inactivation. Normal cell progression can be arrested in a number of ways such as differentiation, mitotic death, interphase death, necrosis, and apoptosis. Apoptosis is an active multistep event for controlling the cell [2]. Cells must have functioning DNA-damage repair and appropriate postrepair processes in order to reach an apoptotic decision point. In contrast to the intuitive expectation of increased apoptotic rates in hypersensitive cells, decreased rates were observed [1, 5, 7, 38]. It should be noted, that cell inactivation and subsequent removal of cells by apoptosis protect the individual against genomic alterations in daughter cells and no late complications can result from these cells. Consequently, increased apoptosis is a favorable process and a decreased apoptosis leads to increased cell problems.

It is possible to measure apoptosis by different specific methods. The most commonly used method is to determine the DNA subpeak in flow cytometry, which is reported to be in good agreement with the TUNEL assay [1, 6]. The TUNEL assay is considered to be highly specific for apoptosis.

Our investigations provide data on spontaneous and radiation-induced apoptosis in Epstein-Barr virus-(EBV-)transformed lymphoblasts and CD^{3+} lymphocytes. A total of 17 lymphoblastoid cell lines (LCLs) were used, derived from healthy donors as well as from individuals with heterozygous and homozygous genotype for AT and NBS. In addition, five patients with a clinically well-documented hypersensitivity against radiation were included, for whom cellular hypersensitivity was documented in advance by FISH analysis of radiation-induced chromosomal aberrations [18]. CD^{3+} lymphocytes of 39 individuals were investigated for radiation-induced

apoptosis, where five patients had a clinical hypersensitivity as well as a proven cellular hypersensitivity to radiation.

Material and Methods

Lymphoblastoid Cells

Lymphocytes were obtained from five healthy donors and transformed into lymphoblastoid cells by EBV [23]. Individual cell lines were examined several times to test the reproducibility of the apoptosis assay. Three EBV-transformed cell lines from AT patients with homozygous and one AT patient with heterozygous genotypes were provided by the Institute for Human Genetics, University of Erlangen-Nuremberg, Germany. Furthermore, cells from two patients with NBS homozygous and one with NBS heterozygous genotypes were EBV-transformed. Distinct increased radiosensitivity was recently found for these cells using a chromosomal aberration assay (three-color FISH) [23]. Five patients with clinical hypersensitivity having late effects of grade 3–4 (RTOG) were identified at the Department of Radiotherapy, University of Erlangen-Nuremberg. All patients were treated with conventional fractionated external radiation using single fractions of 1.8–2.0 Gy and a total dose between 50.4 and 59.4 Gy. No patients with brachytherapy or accelerated treatment were included. There were no preexistent conditions suggesting higher rates of late toxicity. Cells were EBV-transformed and a cellular hypersensitivity was found by FISH. Four of these patients suffered from breast cancer and one patient from rectal cancer. The study with LCLs was carried out in a blinded manner.

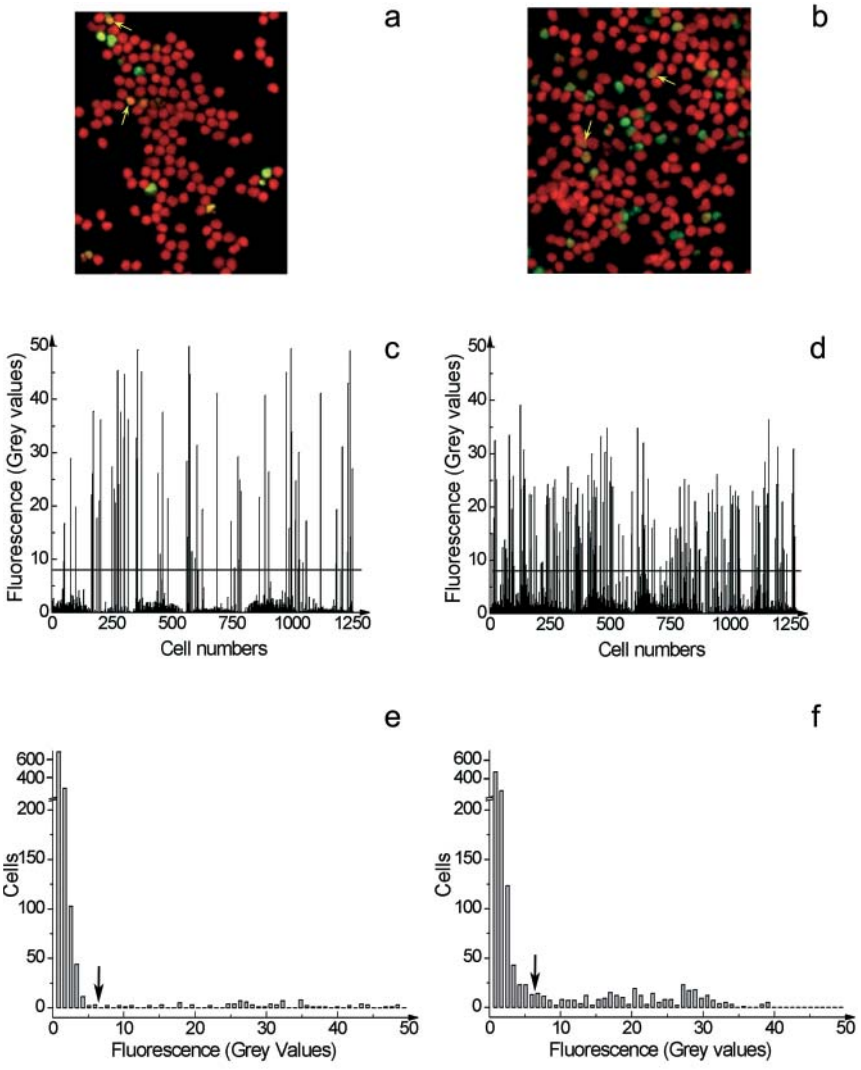
CD^{3+} T-Lymphocytes

Heparinized blood samples were obtained from 21 healthy donors (eleven female and ten male) with an age ranging from 24 to 76 years (mean 47.8 years). For the cancer patient cohort, 18 deidentified samples were received from the Department of Radiotherapy of this University Hospital. Age ranged from 28 to 75 years (mean 55.5 years). The cancer patients (twelve female and six male) suffered from different primary tumors and seven of these showed a significant clinical radiosensitivity of at least grade 3 according to the RTOG late toxicity scale within 1 year after treatment. Clinical radiosensitivity was determined by two independent radiation oncologists. Heparinized (200 µl Liquemin® 2,500 IU, Roche, Basel, Switzerland) blood (10 ml) was drawn from healthy donors and patients after a written informed consent had been obtained. Ficoll separation solution was coated with the blood (17.5 ml Ficoll, Biochrom AG, Germany) and centrifuged about 20 min (300 RCF/decel 0). The cell layer was collected and washed by RPMI.

After centrifugation blood cells were resuspended in 400 µl phosphate-buffered saline (PBS) and 20 µl magnetically labeled antibodies (MACS CD3 MicroBeads, Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated 15 min at 10 °C. Cells were washed by 10 ml PBS, centrifuged at 170 RCF, and the pellet was resuspended in 2.5 ml PBS. The cell suspension including the CD^{3+} cells was applied to an MS^{+} separation column, which was placed in the magnetic field of an MACS separator. The magnetically labeled cells were retained in the column, while the unlabeled cells ran through. After rinsing with 2.5 ml PBS, the column was removed from the magnetic field and a positively selected CD^{3+} cell fraction was flushed out with 5 ml PBS. The cells were centrifuged and distributed into cell culture flasks containing 4 ml medium. Radiation exposure using 120-kV X-rays at room temperature under air with 2 and 5 Gy at a dose rate of 2.2 Gy/min (Isovolt, Seifert, Germany) was followed by incubation at 37 °C in 5% CO_2. After incubating over 48 h, the cell suspension was centrifuged at room temperature. Most of the liquid was removed and the pellets were washed in approximately 15 ml FIX solution (three forths methanol; one forth acetic acid). FIX solution was carefully added to the pellet to avoid lysing the lymphocytes. Cell suspension was partially dropped into a ring formed by a liquid blocker on the slides and after the evaporation of FIX solution slides were preserved in 70% ethanol solution at –20 °C.

For the TUNEL-TdT (terminal transferase nick-end labeling) assay (ApopTag Fluorescein, Oncor, Gaithersburg, MD, USA), the slides were coated first by equilibration buffer (75 µl) for 10 s followed by 110 µl TdT reaction buffer and incubated at 37 °C. After 1 h the slides were washed in distilled water with Stop/Wash Buffer for 15 s and three times in PBS solution for 1 min each. The cells were incubated for 30 min at room temperature in darkness in 65 µl anti-digoxigenin conjugate and after an additional four washes in PBS, the dry slides were coated with 40 µl propidium iodide for 5 min and mounted on Vectashield (Vector Laboratories, Orton Southgate, UK).

Fluorescence was visualized by a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) using a three-band pass filter. Biomas software (Biomas, MSAB, Erlangen, Germany) was used to capture and analyze images acquired by a Hitachi three-chip CCD camera (VH-CC20A, Hitachi Denshi, Rodgau, Germany). Then, the rate of apoptosis was determined by a semiautomatic analysis system. After acquisition of the first of a series of images, thresholds for background reduction and identification of red and green cells had to be identified once. Intensities were extracted and used for automatic background reduction marking the red cells and setting threshold values to identify the total number of cells and green fluorescent cells. The threshold was set by extracting the median gray values of the green spectral band and multiplying the gray scale value by a factor of 1.3. Cells were then automatically marked and could also be deleted or added by user interaction. Counts of total cells and positive green cells were transferred to an Excel sheet (Microsoft Excel 2000, Microsoft Corporation, Redmond, WA, USA) and the fraction of apoptotic cells was calculated. Each slide was analyzed counting a minimum of 1,000 cells.



Figures 1a to 1f. Images of positive selected CD^{3+} lymphocytes from a healthy donor. Spontaneously occurring apoptosis (a) and apoptosis 48 h after irradiation with a dose of 2 Gy X-rays (b) were detected by the TUNEL assay, where red cells (propidium iodide) are nonapoptotic cells and yellow and green cells are apoptotic cells. Cells which were near the cutoff (yellow arrows) and counted as apoptotic cells are labeled. Fluorescence of the green spectral band signal after background reduction of CD^{3+} lymphocytes from a healthy donor is shown. 1,249 cells for analysis of spontaneous apoptosis (c) and 1,279 cells 48 h after an X-ray dose of 2 Gy (d) were counted. a and b are examples of a typical analysis image. To separate nonapoptotic from apoptotic cells, numbers of CD^{3+} lymphocytes with specific fluorescence were extracted from analysis of c and d. A cutoff was computed by the software and indicated by a line, and all cells with a higher fluorescence than seven grey values for the spontaneous (e) and seven grey values for the X-ray-induced apoptosis by a dose of 2 Gy (f) were counted as positive. For example, this case gave 5.6% and 18.1% apoptotic cells.

Abbildungen 1a bis 1f. CD^{3+}-Lymphozyten eines gesunden Spenders mit spontaner Apoptose (a) und strahleninduzierter Apoptose 48 h nach Bestrahlung mit 2 Gy (b). Der Nachweis wurde mit dem TUNEL-Assay durchgeführt, wobei gelbe und grüne Zellen apoptotisch sind und rote Zellen nichtapoptotische mit Propidiumiodid gefärbte Zellen sind. Zellen, die grenzwertig gefärbt sind und noch als apoptotisch gewertet wurden, sind mit gelben Pfeilen markiert. Die Fluoreszenz des grünen Farbkanals nach Hintergrundabzug wird dargestellt (c, d). Für die Analyse der spontanen Apoptose wurden 1249 Zellen (c) und für die durch 2 Gy nach 48 h induzierte Apoptose 1279 Zellen gezählt (d). a und b sind Beispiele von ausgewerteten Bildern. Um nichtapoptotische Zellen von apoptotischen Zellen zu trennen, musste aus c und d die Anzahl der Zellen mit erhöhter Fluoreszenz extrahiert werden. Ein Grenzwert wurde mittels der Software generiert, und alle Zellen mit einer höheren Fluoreszenz als sieben Graustufen, sowohl für spontane (e) als auch strahleninduzierte (f) Apoptose, wurden positiv gewertet. In diesem Fall waren 5,6% und 18,1% der Zellen apoptosepositiv.

Statistical Methods

One-way analysis of variance (ANOVA) was used for evaluating the repeated measurements on the controls and cancer patients and for evaluating intra- and interindividual differences. The apoptosis scores of the patients and the controls were compared using Mann-Whitney U-test. This test was also used to determine differences between male and female donors.

Results

Identification of Apoptotic Cells by Image Analysis

Apoptotic cells were identified by the TUNEL assay, where apoptosis-positive cells were green and cells having no apoptosis were red. Different phases of apoptosis were expressed in different intensity levels of green, seen as yellow cells rising to bright green cells (Figures 1a and 1b). Increased numbers of apoptotic cells were clearly observed at a dose of 2 Gy (Figure 1b). The green spectral band was processed by image analysis software. Background intensity was subtracted and the fluorescence of each cell was determined giving green intensities ranging up to 50 grey values (Figures 1c and 1d). Low and high green intensities indicated nonapoptotic and apoptotic cells. All cells above the cutoff were rated as apoptotic cells (Figures 1e and 1f). Results of automatic counting with user correction of scored cells were compared to direct counting on the microscope and counting cells on the screen by marking cells with the mouse cursor and direct data transfer to an Excel sheet by the count feature of Bio-

Table 1. Apoptotic rates for spontaneous and radiation-induced apoptosis for 15 lymphoblastoid cell lines. Data were compared between repeats of individuals and between groups. AT: ataxia-telangiectasia; CV: coefficient of variation; F: F-distribution; MV: mean value; n: number of individuals; NBS: Nijmegen breakage syndrome; p: significance probability; SD: standard deviation.

Tabelle 1. Spontane und durch 2 Gy induzierte Apoptoseraten in 15 lymphoblastoiden Zelllinien. Die Daten wurden für Wiederholungen und einzelne Gruppen verglichen. AT: Ataxia teleangiectatica; CV: Variationskoeffizient; F: F-Verteilung; MV: Mittelwert; n: Anzahl der Individuen; NBS: Nijmegen-Breakage-Syndrom; p: Signifikanzniveau; SD: Standardabweichung.

				Apoptotic rate			One-way ANOVA Within individuals of a group		Between individuals of all groups		
Cell lines (groups)	Example	n	Dose (Gy)	MV	SD	CV (%)	F	p	n	F	p
Controls	SBL1	5	0	0.082	0.009	11.2	20.2	< 0.001	15	4	< 0.001
			2	0.032	0.010	31.9	2.5	0.114		1	0.281
			5	0.043	0.004	10.1	11.7	0.001		9	< 0.001
AT (homozygous)	p104	3	0	0.045	0.036	81.8	1.0	0.421			
			2	0.004	0.030	795	2.4	0.165			
			5	0.006	0.019	349	7.1	0.020			
NBS (homozygous)	p122	2	0	0.076	0.022	28.9	5.1	0.017			
			2	0.046	0.014	30.4	5.1	0.017			
			5	0.089	0.024	27.0	4.4	0.026			
Patients	p105	5	0	0.036	0.002	5.6	4.8	0.093			
			2	0.049	0.014	27.9	0.0	0.840			
			5	0.090	0.024	26.7	0.2	0.709			

mas. Five counts of 1,000 cells on the screen yielded a deviation of < 1% from automatic counting for both positive and negative cells. Direct counting using a microscope yielded a variation of 3.5% for total cells and 1.5% for positive cells. Automatic counting was at least twice as fast as manual counting.

TUNEL Assay Reproducibility for the Lymphoblastoid Cell Lines

To test the reliability of the TUNEL assay, the interdonor variation was compared to the intradonor variation. Three to four independent measurements from each cell line were taken to determine intradonor variation. The statistical analysis of intradonor, interdonor and intergroup data is given in Tables 1 and 2. A comparison between intra- and interdonor analysis of variance (ANOVA) showed significant differences for all data for the irradiated (5 Gy) lymphoblastoid cells ($p < 0.05$). The intraindividual coefficient of variation (CV) and assay error of control-, NBS- and patient-derived cell lines showed good reproducibility. Only the AT cell lines gave high CV values due to the low apoptotic rates. Between all individuals of all groups, a significant difference was seen for spontaneous apoptosis and after in vitro irradiation with 5 Gy. Rates of spontaneous apoptosis comparing control-, AT-, NBS- and patient-derived cell lines did not show any significant difference (Table 2), however, significant differences ($p < 0.001$) were observed at 5 Gy.

Table 2. Apoptotic rates for spontaneous and radiation-induced apoptosis for 15 lymphoblastoid cell lines. Data were compared between individuals and between groups. AT: ataxia-telangiectasia; CV: coefficient of variation; F: F-distribution; MV: mean value; n: number of individuals; NBS: Nijmegen breakage syndrome; p: significance probability; SD: standard deviation.

Tabelle 2. Spontane und durch 2 Gy induzierte Apoptoseraten in 15 lymphoblastoiden Zelllinien. Die Daten wurden zwischen einzelnen Personen und zwischen den einzelnen Gruppen verglichen. AT: Ataxia teleangiectatica; CV: Variationskoeffizient; F: F-Verteilung; MV: Mittelwert; n: Anzahl der Individuen; NBS: Nijmegen-Breakage-Syndrom; p: Signifikanzniveau; SD: Standardabweichung.

						One-way ANOVA between groups	
Cell lines (groups)	n	Dose (Gy)	MV	SD	CV (%)	F	p
Controls	5	0	0.058	0.023	38.6	1.5	0.230
		2	0.041	0.019	46.0	2.9	0.048
		5	0.054	0.018	33.4	11.6	< 0.001
AT (homozygous)	3	0	0.042	0.025	59.7		
		2	0.012	0.023	189.0		
		5	0.015	0.022	144.3		
NBS (homozygous)	2	0	0.060	0.025	41.1		
		2	0.052	0.065	124.0		
		5	0.093	0.022	23.9		
Patients	5	0	0.045	0.024	54.1		
		2	0.035	0.022	60.9		
		5	0.044	0.033	76.2		

Comparison between Spontaneous and Radiation-Induced Apoptosis in EBV-Transformed Lymphoblasts Derived from Controls and from Patients with Radiosensitivity Syndromes

Five LCLs derived from healthy donors were used as controls (Figure 2a). Four of the cell lines had similar spontaneous rates of apoptosis, while one had a distinctly lower rate. Apoptotic rates observed 48 h after irradiation with 2 and 5 Gy were corrected by subtraction of spontaneous apoptotic rates, giving levels increasing with dose and similar CVs for both spontaneous apoptosis (CV 38.6%) and irradiation with 2 Gy (CV 46.0%) and 5 Gy (CV 33.4%; Table 2).

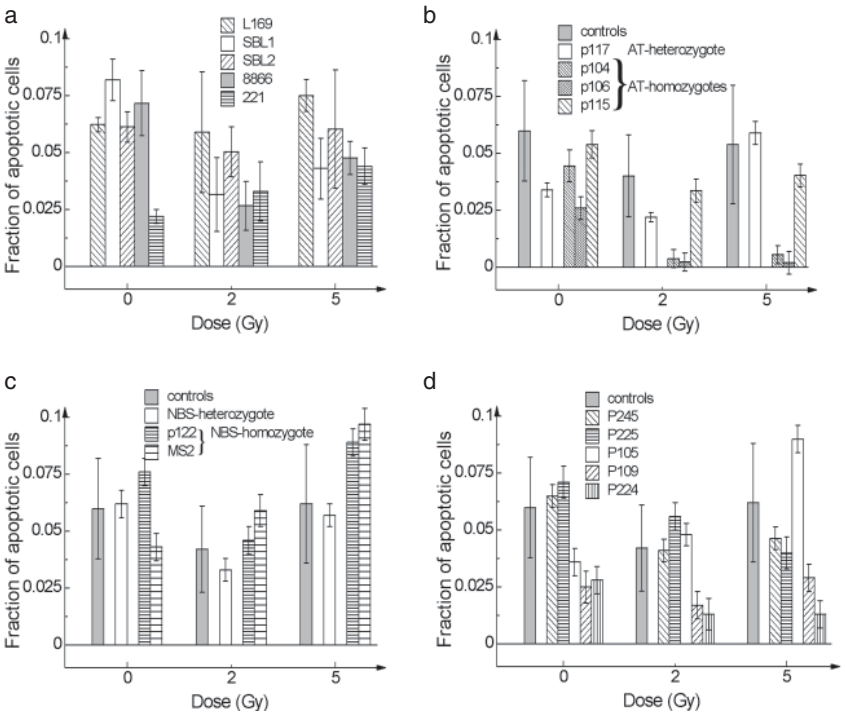
The AT-heterozygous cell line showed comparable apoptotic rates to the control cell lines. LCLs of AT patients had slightly lower spontaneous apoptotic rates (0.042 ± 0.025) than the control group (0.058 ± 0.023; Figure 2b). After subtraction of spontaneous apoptosis, the values of two AT-homozygous cell lines were lower than that of the control cell lines ($p = 0.095$). Mutations in these AT individuals were Q1425X (het), 1561delAG (het; terminal exon 44) and IVS16-1 G-C (het; unknown). A third cell line homozygous for AT with the mutations 5441insT (het) and R2032K (het) led to a frame shift and termination in exon 38 and 44, respectively, and apoptotic rates could not distinguish this cell line from controls ($p > 0.53$). The spontaneous rates of hetero- and homozygous NBS cell lines differed only slightly from controls ($p = 0.61$), however, the 5-Gy values showed a trend to an increase ($p = 0.38$) of the apoptotic rate for the homozygous cell line (Figure 2c). Both cell lines homozygous for NBS had the mutation 675del5.

Comparison between Spontaneous and Radiation-Induced Apoptosis in Controls and Cancer Patients

Five LCLs from cancer patients with severe late toxicity (RTOG ≥ grade 3) after standard radiotherapy were retrospectively identified. These LCLs and peripheral blood lymphocytes from the same patients were previously identified by three-color FISH to have an increased cellular radiosensitivity. After in vitro irradiation two of these cell lines showed reduced levels ($p = 0.095$) compared to controls (Figure 2d).

CD^{3+} Lymphocytes of Healthy Controls and Cancer Patients

CD^{3+} lymphocytes from 21 healthy donors were tested as a matched-pair analysis together with 18 patients consisting of five, four and one patient with head and neck, breast and rectal cancer, respectively. The other eight patients had different tumors. There was no correlation with sex when the rates of spontaneous and radiation-induced apoptosis were compared between controls ($p = 0.113$) and patients ($p = 0.190$). However, age of control individuals showed a decreasing radiation-induced apoptosis rate by 0.16% $year^{-1}$ at 2 Gy and 0.15% $year^{-1}$ at 5 Gy (Figure 3). Therefore, apoptotic rates were cor-



Figures 2a to 2d. Spontaneous and radiation-induced apoptotic rates of five LCLs from healthy donors (a). Apoptotic rates of LCLs with AT-heterozygous (open bars) and AT-homozygous (striped bars) genotype in comparison to the average of five cell lines from healthy donors (filled bars) (b). Apoptotic rates of LCLs with NBS-heterozygous (open bar) and NBS-homozygous (striped bars) genotype compared to the average of five cell lines from healthy donors (filled bars) (c). Apoptotic rates of five LCLs (stripped and open bars) derived from cancer patients who have suffered from severe treatment-related ≥ grade 3 (RTOG) toxicity compared to the average of five cell lines from healthy donors (filled bars) (d). Spontaneous apoptotic rates were subtracted from radiation-induced rates. Each data point is derived from three independent experiments counting 1,000 cells. Error bars indicate the standard deviation.

Abbildungen 2a bis 2d. Spontane und strahleninduzierte Apoptoseraten von fünf LCL von Gesunden (a), von LCL mit AT-heterozygotem (weiße Balken) und AT-homozygotem (gestrichelte Balken) Genotyp im Vergleich zum Durchschnitt von fünf LCL von Gesunden (graue Balken) (b), von LCL mit heterozygotem (weiße Balken) und homozygotem Genotyp (gestrichelte Balken) für NBS im Vergleich zu Gesunden (graue Balken) (c). Apoptoseraten von fünf LCLs (gestrichelte und weiße Balken) von Krebspatienten mit therapiebedingten Strahlennebenwirkungen ≥ Grad 3 (RTOG) im Vergleich zu Gesunden (d). Spontane Apoptoseraten wurden von strahleninduzierten Apoptoseraten abgezogen. Jeder Datenpunkt stammt von drei unabhängigen Versuchen mit 1000 gezählten Zellen pro Versuch. Fehlerbalken stellen die Standardabweichung dar.

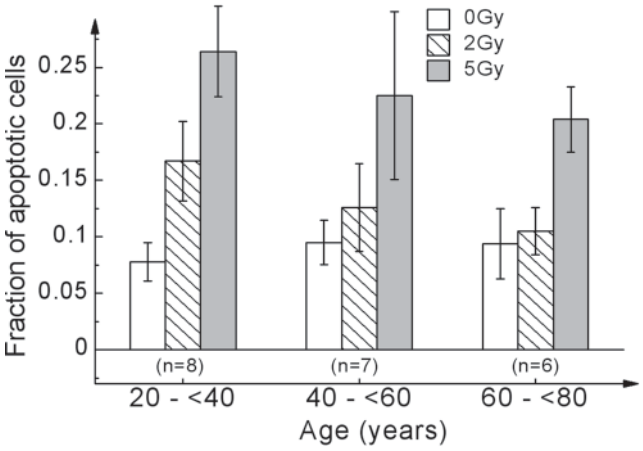


Figure 3. Effect of age on apoptotic rates of the CD^{3+} cells from controls grouped into 20 to < 40 years, 40 to < 60 years, and 60–80 years. Cells were grown 48 h after irradiation with 2 Gy (striped bars) and 5 Gy (filled bars) and the apoptotic rates were analyzed by the TUNEL assay. Spontaneous apoptotic rates were subtracted from radiation-induced rates. Error bars indicate the standard deviation.

Abbildung 3. Altersabhängigkeit der Apoptoseraten in CD^{3+}-Zellen bei Gesunden im Alter zwischen 20 bis < 40 Jahren, 40 bis < 60 Jahren und 60–80 Jahren. Die Zellen wurden mit 2 Gy (gestrichelte Balken) und 5 Gy (graue Balken) bestrahlt und die Apoptoseraten mit dem TUNEL-Assay gemessen. Spontane Apoptoseraten wurden von strahleninduzierten Apoptoseraten abgezogen. Fehlerbalken stellen die Standardabweichung dar.

rected for the decline with increasing age by a value of 0.15% per year. For comparative purposes, 0.15% was added for patients > 55 years and subtracted for younger patients (Figure 4). A marked difference between both groups, controls and patients, was seen for the CV. Apoptotic values of controls appeared more homogeneous than that of the hypersensitive patients. The difference of CV existed between spontaneous ($CV_{controls} = 28.7\%$; $CV_{patients} = 42.6\%$) as well as radiation-induced (5 Gy) apoptosis ($CV_{controls} = 24.0\%$; $CV_{patients} = 89.9\%$), irrespective of the applied radiation doses.

Classification of Cancer Patients into Groups with Different Radiosensitivity

Apoptotic levels of radiosensitive cancer patients were compared to patients without considerable early or late normal-tissue reactions (Figure 4). Seven patients had suffered from grade 3, three from grade 2 and seven from grade 1 toxicity (RTOG). Five of the seven patients with ≥ grade 3 toxicity had previously been tested and identified by cytogenetic analysis (FISH) to have an elevated radiosensitivity [17, 18]. Only two of the five patients had distinct reduced apoptotic rates including one of the two additional patients with grade 3 toxicity. Two patients with grade 2 toxicity had reduced apoptotic rates, while all patients with grade 1 toxicity had higher levels, yet markedly reduced in comparison to controls ($p < 0.08$). Lack of homogeneity of the sensitive patient values was in remarkable contrast to the seven patients with grade 1 toxicity and controls which showed very similar values.

Influence of Previous Radiotherapy and Chemotherapy

Apoptotic rates for unirradiated cells from controls and patients were similar ($p = 0.59$). After irradiation with 2 Gy ($p = 0.002$) and 5 Gy ($p = 0.003$), apoptotic rates for cancer patients were lower than controls. Spontaneous apoptotic rates in cells from patients without previous irradiation (6.9% ± 1.4%) were lower compared to patients who had undergone radiotherapy (10.6% ± 3.2%; $p = 0.055$). Cells derived from prospectively evaluated patients did not show different apoptotic values compared to those from previously irradiated cancer patients ($p > 0.33$). Cells from cancer patients with ($n = 5$) and without ($n = 13$) previous chemotherapy showed very similar spontaneous apoptotic rates ($p = 0.14$) and similar rates after in vitro irradiation with 2 Gy ($p = 0.9$) and 5 Gy ($p = 0.57$).

Discussion

Radiation-induced apoptosis of in vitro irradiated LCLs and CD^{3+} lymphocytes from patients were studied to evaluate whether patients with radiation hypersensitivity can be identified with this analysis endpoint. Results from LCLs showed, that only two of the three cell lines homozygous for AT showed low apoptotic rates. The third had a similar rate and the two cell lines homozygous for NBS showed a higher apoptotic rate compared to controls. Similar results were seen in our patients group studied with CD^{3+} lymphocytes. Only two of the five patients with a clinically detected severe toxicity (RTOG ≥ 3) and cytogenetic proof of elevated cellular radiosensitivity could be identified by lowered apoptotic rates.

Previous studies on radiation-induced apoptosis as a predictor of individual radiosensitivity have provided conflicting results [1, 4–8, 31, 37, 38]. The apoptosis detection method might be of crucial importance. Apoptosis can be measured by changes of different morphological and biochemical characteristics. Early events like the translocation of phosphatidylserine from the cytoplasmic surface of the cell membrane to the external cell surface may be detected by the annexin V protein [37]. Changes in cell morphology, including convoluted budding and blebbing of the membrane, are characteristic of apoptosis. Frequently, fragmentation of the DNA is measured by agarose electrophoresis [21], Comet assay [38], TUNEL assay [1], or flow cytometry [4, 5, 7, 8]. The TUNEL assay is based on the action of the endogenous enzyme DNase I which is activated in response to apoptotic signals creating typical 3'-OH ends in the fragmented DNA. Terminal deoxynucleotidyl transferase binds to 3'-OH ends and catalyzes the addition of indirect- or direct-labeled deoxynucleotides. This is a standard technique and is compared to other faster and less expensive methods like flow cytometry detecting DNA content and morphology (by light scattering)

of the cells [1, 5, 7]. Our approach was to use a fluorescence microscope in combination with a semiautomatic image analysis system to visualize TUNEL staining together with cell morphology.

Another crucial point in testing radiosensitivity is the choice of the most suitable cell population. It is well known that radiosensitivity is dependent on cell cycle and therefore restricts the utilization of fibroblasts to quantify radiosensitivity by the clonogenic assay to cells in G1/G0 [10]. Additionally, the progression into a differentiated state by cell culture-induced stress makes the detection of individual radiosensitivity difficult [11]. The advantage of having peripheral blood mononuclear cells in G0 has to be compared to the disadvantage represented by different subpopulations like granulocytes, B-cells, natural killer (NK) cells, and T-cells. This problem may be easily overcome by labeling for specific markers or by selecting cells using magnetic beads [38]. It has been shown, that different subpopulations of peripheral blood mononuclear cells have different spontaneous and radiation-induced apoptotic rates [31, 38]. Granulocytes and B-lymphocytes have high spontaneous apoptotic rates. Granulocytes have lower inducible apoptosis as related to B-cells which have 20–30% radiation-inducible apoptotic rates. Quite homogeneous spontaneous rates are found for $CD4^+$ and $CD8^+$ cells [38], while $CD8^+$ cells have higher apoptotic rates than $CD4^+$ cells [30, 38]. Our approach was twofold, first, to use LCLs derived from B-cells, and second, to separate mononuclear cells by density gradient centrifugation followed by isolation of the $CD3^+$ cells by magnetic beads. CD3 antibody recognizes all T-cells which means it reacts with 70–80% of human peripheral blood lymphocytes and 65–85% of thymocytes. Consequently, CD3 antibodies identify all cells detected by CD4 and CD8 antibodies.

Other parameters that may influence apoptosis include culture conditions and cell concentration in the medium after irradiation. The culturing of whole blood [22] or all mononuclear cells [1] together and using flow cytometry methods by labeling the cells may have an advantage over the cell separation and culturing relatively small numbers of cells of a homogeneous subpopulation. According to our experience, culturing cells in conical tubes with a high cell density and sufficient nutrients can achieve a stress-free [27] cultivation of the isolated $CD3^+$ cells.

It is important for a radiosensitivity assay to have a high level of reproducibility and validity. Reproducibility can be demonstrated by testing cells of a patient several times and comparing the intradonor variability with the interdonor variability. This was performed using LCLs, because of the option to evaluate multiple cell lines and to perform numerous replicate experiments. The intradonor variation was smaller than the interdonor variation, as shown by others [1, 6]. Additionally, our results indicated that apoptotic rates yielded a better discrimination of patients following irradiation using 5 Gy as compared to 2 Gy.

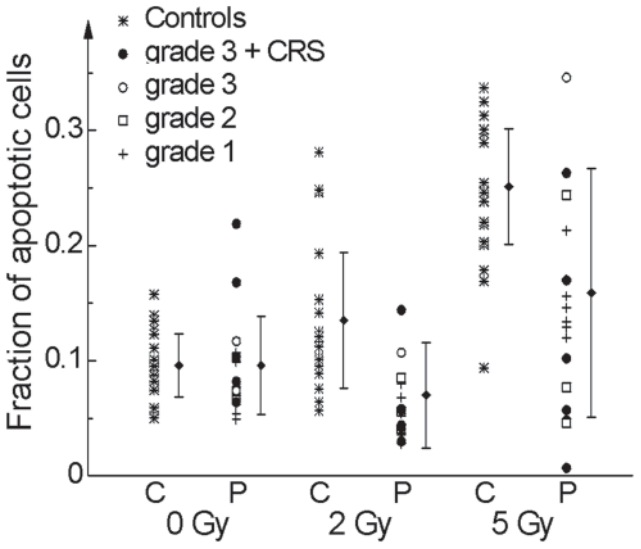


Figure 4. Apoptotic rates of CD^{3+} lymphocytes from 21 healthy donors (C, ✳) and 18 cancer patients (P) with different kinds of tumors. Three groups of patients which suffered from grade 1 (+), 2 (□) and 3 (○) toxicity after radiotherapy were graphed. Another group were patients with grade 3 toxicity which were previously identified by cytogenetic analysis to have a cellular elevated sensitivity (●, CRS). Error bars show the standard deviation of healthy donors or cancer patients (◆). Cells were grown 48 h after irradiation with 5 Gy and the apoptotic rates were analyzed using the TUNEL assay. Spontaneous apoptotic rates were subtracted from radiation-induced rates.

Abbildung 4. Apoptoseraten von CD^{3+}-Lymphozyten bei 21 Gesunden (C, ✳) und 18 Krebspatienten (P) mit unterschiedlichen Tumoren. Davon litten Patienten nach Strahlentherapie an Nebenwirkungen Grad 1 (+), 2 (□) und 3 (○). Eine weitere Gruppe von Patienten mit Grad-3-Nebenwirkungen war zuvor durch zytogenetische Untersuchung getestet und als zellulär strahlenempfindlich gewertet worden (●, CRS). Fehlerbalken stellen die Standardabweichung bei Gesunden und Patienten (◆) dar. Die Zellen wurden 48 h nach Bestrahlung mit 5 Gy mittels des TUNEL-Assays auf Apoptose getestet. Spontane Apoptoseraten wurden von strahleninduzierten Apoptoseraten abgezogen.

Validity of a test system is much more difficult to estimate, since it is difficult to determine whether a clinically detected severe side effect is due to factors related to the treatment itself or whether it is based on a genetic disorder leading to cellular radiation hypersensitivity. One important indicator for the validity of our apoptosis test system is that we found a decrease in inducible apoptosis with age which has previously been observed by other investigators [7]. The ability to confirm the decrease of apoptosis with age is greatly dependent on the method used and on the subpopulation of lymphocytes. Some studies failed to show a negative unequivocal correlation with increasing age [1, 38]. There was a decrease in apoptosis varying from 0.15% $year^{-1}$ for $CD8^+$ and 0.3% $year^{-1}$ for $CD4^+$ at 2 Gy to 0.43% $year^{-1}$ for $CD8^+$ and 0.65% $year^{-1}$ for $CD4^+$ at 9 Gy [8]. We have found a decreased apoptotic rate of 0.15% $year^{-1}$ ($CD3^+$), which corresponds exactly to the apoptotic rate after 2 Gy in $CD8^+$ cells reported by Crompton et al.

[8]. Additionally, our apoptotic rate of 13% for $CD3^+$ cells from 50-year-old individuals correlated quite well with that of the $CD4^+$ and $CD8^+$ cells at 2 Gy where apoptotic rates of 10% (CD4) and 18% (CD8) were reported [8].

Five LCLs derived from healthy normal individuals were used as controls. The cell line L169 was checked and found to be free of mutations in the AT gene, while others were presumed to be normal. One cell line had a low spontaneous apoptotic rate, while induced apoptotic rates were quite homogeneous. In contrast to blood samples from individuals with a homozygous phenotype for AT [1], spontaneous rates were lower than that of the controls. In a previous study, cellular radiosensitivity of the AT cell lines used in this study was evaluated by three-color FISH, a more demanding method, indicating radiosensitivity with a high sensitivity [18, 23, 24]. Control cell lines showed 0.4 ± 0.24 breaks per metaphase (b/m) after 48 h following irradiation with 2 Gy [23] (code no. 14 in Tables 1 to 3 of the publication [23]), while the AT-heterozygous cell line had 1.03 b/m (2 Gy) characterized by a 5441insT frame shift mutation leading to truncation. The two cell lines with almost no apoptotic response to irradiation had chromosomal breakage rates of 2.6 and 1.46 b/m (2 Gy; code no. 1 and 2). The third AT-homozygous line with the frame shift mutation, 5441insT (het), and the splice site mutation, R 2032 K (het; code no. 11), had a high chromosomal radiation sensitivity of 2.82 b/m, however, the apoptotic fraction was reduced to a value similar to the controls after radiation. It clearly shows that a highly radiosensitive cell line could not be identified as radiosensitive by a reduction in the apoptotic rate after radiation.

Very similar results were obtained with two NBS cell lines, which had comparable high cellular radiosensitivity (1.47 and 1.83 b/m) like AT cells as determined by chromosomal aberrations [23], yet they exhibited similar or slightly higher apoptotic rates than the controls. Both cell lines had the mutation 657del5 in exon 6 in both alleles of the NBS1 gene leading to formation of a truncated version of the protein nibrin. It is the typical founder mutation in NBS [35]. In contrast to our findings, another study investigating $CD4^+/CD8^+$ cells derived from an NBS-homozygous patient found a reduced apoptotic rate when compared to the average control cell rate [7].

Five cell lines from patients with severe treatment-related side effects were identified to have increased cellular radiosensitivity as determined by the FISH assay, but only two of these had a distinctly lower apoptotic rate. We conclude that it is possible to detect increased radiosensitive cells by low apoptotic rates in LCLs. Nevertheless, there are sensitive cell lines, which cannot be identified by this approach.

Increased radiosensitivity is an event, which can be caused by mutation in different genes. This can be seen by the various genomic instability syndromes. Radiosensitivity is increased in Bloom's syndrome, Werner's syndrome and Rothmund-Thomson syndrome by mutations in different genes encoding DNA helicases [9, 26]. Mutations in eight different genes can lead to Fanconi's anemia (FA) [3, 9]. One FA form is caused by a BRCA2 mutation [15]. AT-mutated protein functions as the central controlling unit of the cell in response to DNA damage [20]. There are a number of proteins which are phosphorylated by Atm leading to their activation or inactivation. Cellular radiosensitivity and severity of the AT disease [32] are dependent on different locations and types of mutations [23] and cancer predisposition [32] may result. Mutations in the NBS gene, which is involved in DNA repair, lead to a high radiosensitivity similar to AT mutations [13]. Recently, mutations in two different genes, MRE11 and ligase IV, involved in DNA repair have been identified for individuals who had AT-like diseases. Mutations in the MRE11 gene leads to abrogation of Mre11-Rad50-Nbs1 radiation-induced foci [33] and a mutation in ligase IV disrupts the ligase IV domain or the interaction of the ligase IV and Xrcc4 [25, 28]. There are > 130 other repair proteins [39] and many more proteins in the entire DNA-damage response system which include cell-cycle control and tumor suppressor proteins. A mild loss of function of these proteins could play a part in an inadequate DNA-damage response causing hypersensitivity to ionizing radiation [36]. However, in patients hypersensitive to radiation, a reduced induction of apoptosis may only result from alterations in specific pathways involved in apoptotic response. Alternatively, another mechanism of cellular inactivation such as permanent cell-cycle arrest may be triggered. As these investigations have shown, even AT cells can have an intermediate apoptosis induction and NBS cells can have a normal apoptotic rate. It can be speculated, that if hypersensitivity is caused by mild mutations in the DNA-damage response system, only a subgroup will have reduced apoptotic rates depending on the involvement in the apoptosis-inducing pathways.

Conclusion

These data indicate that measurement of radiation-induced apoptosis is not suitable to detect individual radiosensitivity. Only a subgroup of patients with increased radiosensitivity showed reduced apoptotic rates. However, the apoptotic pathway is only one process that may affect or be affected by radiosensitivity. Knowledge of the apoptotic status could help elucidate underlying mechanisms of radiosensitivity at least in some cases. Therefore, it is necessary to combine more than one method for the detection of individual radiosensitivity including assessments of remaining DNA double-strand breaks, apoptosis, cell-cycle control and measurement of chromosomal aberrations.

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