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Differential Role of DNA-PKcs Phosphorylations and Kinase Activity in Radiosensitivity and Chromosomal Instability

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Abstract

The catalytic subunit of DNA-dependent protein kinase (DNA-PKcs) is the key functional element in the DNA-PK complex that drives nonhomologous end joining (NHEJ), the predominant DNA double-strand break (DSB) repair mechanism operating to rejoin such breaks in mammalian cells after exposure to ionizing radiation. It has been reported that DNA-PKcs phosphorylation and kinase activity are critical determinants of radiosensitivity, based on responses reported after irradiation of asynchronously dividing populations of various mutant cell lines. In the present study, the relative radiosensitivity to cell killing as well as chromosomal instability of 13 DNA-PKcs site-directed mutant cell lines (defective at phosphorylation sites or kinase activity) were examined after exposure of synchronized G₁ cells to ¹³⁷Cs γ rays. DNA-PKcs mutant cells defective in phosphorylation at multiple sites within the T2609 cluster or within the PI3K domain displayed extreme radiosensitivity. Cells defective at the S2056 cluster or T2609 single site alone were only mildly radiosensitive, but cells defective at even one site in both the S2056 and T2609 clusters were maximally radiosensitive. Thus a synergism between the capacity for phosphorylation at the S2056 and T2609 clusters was found to be critical for induction of radiosensitivity.

INTRODUCTION

DNA double-strand breaks (DSBs) are the principal lesions responsible for the major biological effects of radiation. Such DSBs can be repaired in mammalian cells by at least two major pathways: nonhomologous end joining (NHEJ), which operates throughout the cell cycle, or homologous recombination repair (HRR), which operates during S or G₂ (1). The NHEJ pathway uses several enzymes that capture both DNA ends and bring them together in a synaptic complex to facilitate direct ligation of the DNA break (2). One of the first enzymes to be attracted to DSBs is the Ku70/80 heterodimer; subsequently, the DNA-Ku70/80 scaffold recruits a large 460-kDa serine/threonine kinase called the DNA-

dependent protein kinase catalytic subunit (DNA-PKcs). The protein complex formed after the association of both Ku70/80 and DNA-PKcs at the DNA ends is generally referred to as the DNA-dependent protein kinase (DNA-PK). DNA-PK kinase activity was shown to be dependent on the functional Ku protein (3,4). The xrs-6 cells defective in Ku DNA binding lack DNA-PK activity and DSB repair, both of which can be restored in xrs-6 cells by introducing wild-type Ku80 (3).

A greater degree of radiosensitivity has been reported in terms of the extent of DNA repair, cell killing and chromosomal aberrations in Ku-deficient cells than in DNA-PKcs-deficient cells (5-7). In addition, Ku70/80-deficient xrs-5 and -6 cells have been reported to have greatly reduced cellular repair throughout cell cycle, no radiation dose-rate effect, and no repair of potentially lethal damage (8). The cell cycle effect in particular appears to be significantly different in Ku70/80-deficient cells and DNA-PKcs-deficient cells (9-14).

Several activities of DNA-PKcs have been shown to contribute its function in DSB repair, including the intrinsic kinase activity and phosphorylation. The kinase activity of DNA-PKcs is essential for DSB repair (15), likely through phosphorylation and regulation of NHEJ factors including DNA-PKcs itself (2). Thus far, many phosphorylation residues of DNA-PKcs have been identified both *in vitro* and *in vivo* (16-22). Most of the DNA-PKcs phosphorylation sites are in the S/TQ motifs (serine or threonine followed by a glutamine residue) commonly present in many DNA damage repair proteins and are the cognate substrates of PIKK kinases (23). In addition, the critical phosphorylation residues of DNA-PKcs are largely concentrated at the T2609 and the S2056 clusters (16-20). Although the mechanism for DNA-PKcs phosphorylation remains to be clarified, as is the case for its kinase activity, DNA-PKcs phosphorylation is required for DSB repair.

The present study was designed to measure and compare the relative radiosensitivity of G₀/G₁ synchronized site-directed mutant cells involving phosphorylation residues of the T2609 cluster (16-18), the S2056 cluster (19,20), and the carboxyl-terminus PI3K domain (15) of DNA-PKcs. Radiosensitivity examined in asynchronous randomly dividing cell populations may be dependent on the cell line because it represents the average response of a mixture of cells in different phases of the cell cycle, particularly of cells in the most radioresistant phases (24-26). In such instances the contributions of other processes such as HRR greatly complicate the interpretation of results. We have thus chosen to study synchronized cell populations in which repair will occur primarily via the NHEJ pathway to reduce the confounding factor of differences in cell cycle distribution among different cell lines.

MATERIALS AND METHODS

Cell Lines and Cell Culture

For these studies, we employed the wild-type Chinese hamster cell lines CHO (27) and AA8 (28), NHEJ-deficient xrs-5 cells (29) and V3 cells (10), and cell lines derived from DNA-PKcs null V3 cells with complemented human DNA-PKcs cDNA containing amino acid substitutions at various positions that are described in Fig. 1 and Table 1 (15,16,18,19). The cells were maintained at 37°C in a humidified 95% air/5% CO₂ atmosphere in Eagle's minimal essential medium (MEM, containing 52 mg/liter isoleucine) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (50 µg/ml), and streptomycin (50 mg/ml). When the cultures approached 30% confluence in T-25 tissue culture flasks, the normal growth medium was removed. The medium was then changed twice at 24-h intervals to isoleucine-deficient MEM containing 5% 3× dialyzed fetal bovine serum to synchronize the cells in G₀/G₁ phase. The experiments were initiated 1 day after the second medium change. At this time the cells were synchronized in G₀/G₁ phase (30). Cells were pulse labeled with

30 μ M BrdU for 15 min and then fixed in 70% ethanol. Fixed cells were treated with 0.1 mg/ml RNase A and 3N HCl sequentially for 30 min at 37°C, then incubated with BrdU antibody conjugated with Alexa Fluor 488 (Invitrogen) for 2 h at 37°C. The cells were analyzed using a FACScan flow cytometer and CellQuest software (BD Biosciences). Although more than 10% S-phase cells were observed in some of the cell lines, others showed many fewer S-phase cells after IL-deficient synchronization as shown in Table 2. The mean doubling time was determined from the linear portion of the growth curves of the exponentially growing cell population.

Irradiation and Colony Formation

Synchronized G₀/G₁ cells were irradiated aerobically at room temperature. The radiation source was a J. L. Shepherd and Associates irradiator that emitted ¹³⁷Cs γ rays at a dose rate of 2.5 Gy/min. Survival curves were obtained by measuring the colony-forming ability of irradiated cell populations. Cells were plated postirradiation onto 100-mm plastic petri dishes and incubated for 7–10 days for colony formation. The dishes were then fixed with 100% ethanol and stained with 0.1% crystal violet solution. A colony with more than 50 cells was scored as a survivor.

Chromosome Analysis

Exponentially growing cells were irradiated with ¹³⁷Cs γ rays at a dose rate of 2.5 Gy/min. Colcemid was added to a final concentration of 1 μ g/ml starting at 30 min after irradiation, and the cells were harvested 4 h later so that the mitotic cells collected would have been in late-S/G₂ phase at the time of irradiation (48). Mitotic cells were harvested by trypsinization and hypotonic treatment. Cells were fixed in methanol:acetic acid (3:1) and chromosomes were spread by air-drying (31). After the slides were stained with Giemsa, chromosome aberrations were scored.

RESULTS AND DISCUSSION

In a study using asynchronous exponentially growing cells, it was reported that DNA-PKcs cell lines containing site-directed mutation at the S2056/T2609 clusters as well as the C-terminal phosphatidylinositol 3-kinase (PI3K) kinase domain were radiosensitive (15–20). The synchronized DNA-PKcs mutant cell lines irs-20 and V3 were very radiosensitive when irradiated in the G₁ phase (nearly the same as Ku80-deficient xrs-5 cells) but displayed rapidly increasing cell survival (decreasing radiosensitivity) when they were irradiated throughout S phase, peaking at late S phase and then declining (13,19,32). Because other DNA repair processes such as HRR also contribute to survival but operate virtually exclusively during S and G₂, it is important to study radiosensitivity with synchronous G₁-phase cells for proper interpretation of results. The present study was performed with cells incubated in isoleucine-deficient medium to synchronize the cell populations in G₀/G₁ phase (30). The radiosensitivity of cells synchronized in G₁ by isoleucine deprivation is similar to that of cells synchronized in G₁ by allowing harvested mitotic cells to progress into G₁ (33).

The experiments were carried out after all cell line designations were coded blindly as lines 1–12 and 14 without knowing the genotype of each cell line. Radiation effects on survival and chromosomal aberrations were examined in the site-directed mutant cell lines with mutations at the S2056 cluster, the T2609 cluster and the PI3K domain (Table 1 and Fig. 1). Table 3 shows cell killing after irradiation in G₀/G₁ as well as radiation-induced total chromosomal aberrations in late S/G₂-phase cells among the various Chinese hamster cell lines compared to the DNA-PKcs site-directed mutant cell lines. We measured the D_0 and D_{10} from complete survival curves for determining relative radiosensitivity as described in

the last column and footnotes in Table 3. These survival curves are shown graphically in Fig. 2.

Both the L-2 and L-3 cell lines contain the mutated T2609 cluster, in which all six serine/threonine sites were replaced with alanine (A). When irradiated in the G_0/G_1 phase, these cell lines were very radiosensitive and fell in the same shaded area on the dose-survival plot as xrs-5 and V3 cells (L-7 empty vector) (Fig. 2A). L-14 cells also containing a mutated T2609 cluster with three threonine residues were replaced with alanine (T2609A/T2638A/T2674A) and showed intermediate radiosensitivity, but they were more radiosensitive than L-6 cells, in which only a single amino acid was replaced (T2609A) (Fig. 2A).

The single-site replacement of alanine in L-5 (S2056A) and L-6 (T2609A) mutant cell lines resulted in only mild hypersensitivity to radiation. However, L-4 (S2056A/T2609A) cells displayed synergistically increased radiosensitivity compared to cell lines with complete loss of the T2609 cluster (L-3) or complete loss of the S2056 cluster. The hyper-radiosensitivity of L-4 cells in G_0/G_1 is distinctive from the mild radiosensitivity when exponentially growing cells were irradiated, whereas L-5 (S2056A) cells showed similar radiosensitivity to γ rays in G_0/G_1 phase or in asynchronous conditions (18,19).

It has been reported that the measurement of NHEJ direct end joining relative to alternative microhomology-directed end-joining activity increased dramatically in cells defective in NHEJ compared to the normal cell lines (34). Although there was no significant difference in ionizing radiation-induced cell killing between S2056A (L-5) and S2056A/T2609A (L-4) cells when they were irradiated in asynchronous cultures, measurement of microhomology-directed end joining showed a significant increase (~80%) in S2056A/T2609A (L-4) cells as well as in V3 (L-7) cells, whereas S2056A (L-5) and T2609A (L-6) cells showed less induction (~60%) of microhomology-directed end joining (19). The difference in microhomology-directed end joining suggests that NHEJ repair is severely compromised in L-4 (S2056A/T2609A) cells, reflecting the hyper-radiosensitivity of L-4 cells irradiated in G_0/G_1 . The results also implied that the two sites (S2056 and T2609) may have some kind of interaction or synergistic effect on radiosensitivity. It is not clear why there are large differences in sensitivity for radiation-induced cell killing between L-4 and L-5/L-6 cells. Although the S2056 and T2609 clusters are 553 amino acids apart (Fig. 1), this does not exclude a closer location that depends on the three-dimensional structure of DNA-PKcs leading to synergistic interaction that could increase radiosensitivity in the L-4 cell line.

The kinase activity of DNA-PKcs is essential for NHEJ repair because kinase-dead DNA-PKcs mutant cell lines (L-8, -9, -10 and -11) with site-directed mutations within the conserved PI3K domain significantly lowered DNA-PKcs kinase activity and increased radiosensitivity, even though they contained different amino acid substitutions or truncation (15). All four kinase-dead mutant cell lines were very sensitive when the cells were irradiated in G_0/G_1 phase (Table 3 and Fig. 2B) and displayed five to ten times greater radiosensitivity in G_0/G_1 phase than in asynchronous cell populations with 2 Gy (15).

In addition to investigating radiosensitivity of each mutant cell lines in G_0/G_1 , we found that aneuploidy occurred in several DNA-PKcs mutant cell lines, as shown in Table 2. The L-2 and L-3 cell lines, in which all six phosphorylation residues of the T2609 cluster were substituted to alanine, were both very radiosensitive and aneuploid. Aneuploidy was also found in L-4 (S2056A/T2609A) cells but not in the single-site mutated L-5 (S2056A) and L-6 (T2609A) cell lines. On the other hand, two L-12 cell lines (L-12-10, L-12-15) in which all five putative phosphorylation residues of the S2056 cluster were substituted to alanine showed only mild radiosensitivity in G_0/G_1 phase (Table 3 and Fig. 2A) but displayed severe aneuploidy (Table 2). All four kinase-dead mutant cell lines with different types of

mutations at the PI3K domain were very radiosensitive (Table 3 and Fig. 2B), but only the L-9 cell line was aneuploid (Table 2). While there was no direct cause-and-effect relationship seen between aneuploidy and radiosensitivity, it cannot be ruled out that certain DNA-PKcs mutations can lead to a propensity to develop aneuploidy.

Total spontaneous chromosomal aberrations as well as chromosomal aberrations induced by 0.5 Gy γ rays in G₂ were analyzed in wild-type (CHO and AA8) and NHEJ mutated cells (Ku 70/80 deficient xrs-5 and DNA-PKcs site-directed mutant cell lines). There were higher frequencies of spontaneous chromosomal aberrations in NHEJ mutated cells than in wild-type CHO and AA8 cells (Table 3). This may suggest that these mutant cell lines developed genetic instability and/or aneuploidy during the process of establishing stable mutant clones (Tables 2 and 3). An assay of radiation-induced G₂ chromosomal aberrations was performed by irradiating exponentially growing cells followed by 4 h of Colcemid treatment starting 30 min after irradiation. Mitotic cells collected under this protocol would have been in late S/G₂ phase at the time of irradiation (48). The G₂-phase chromosomal assay has been applied to number of radiosensitivity and cancer predisposition syndromes (35-38). Chinese hamster cells deficient in either NHEJ or HRR showed similar increases in radiation-induced chromosomal aberrations in late S/G₂ phase (39-42). After 0.5 Gy of γ rays, total chromosomal aberrations in late S/G₂-phase cells were also significantly elevated for all NHEJ mutant cell lines compared with the wild-type CHO and AA8, except the L-5 and L-6 cell lines (Table 3). These two cell lines showed near normal radiosensitivities and near diploid chromosome numbers (Tables 2 and 3). It is notable that L-12 cells, which contain the mutated S2056 cluster, in which all five serine sites were replaced with alanine, displayed a high frequency of spontaneous and radiation-induced chromosomal aberrations, although L-12 cells showed only mild radiosensitivity in G₀/G₁ phase (Table 3 and Fig. 2A).

The relationship between DNA-PKcs activity and DSB repair underlying the NHEJ mechanism has been widely studied. Results from the current study provide further insight into the contributions of different modifications of DNA-PKcs activity (phosphorylations and kinase activity) to radiosensitivity phenotypes in G₀/G₁-phase cells. Mice deficient in DNA-PKcs and NHEJ components have been characterized by increased sensitivity to agents causing DNA damage as well as to chromosomal instability, immunodeficiency and predisposition to thymic lymphomas (43). Studies of clinical samples have also correlated DNA-PKcs activity with cancer risk and prognosis. A reduction of DNA-PKcs expression or DNA-PK kinase activity in peripheral blood lymphocytes (PBL) has been found in bronchial epithelial cells (a progenitor cell for lung cancer) (44) and was associated with a risk for breast and uterine cervix cancer as well as an increased frequency of chromosomal aberrations (45). Negative expression of DNA-PKcs has also been correlated with tumor progression and poor patient survival in gastric cancer (46). Recently, several point mutations of DNA-PKcs have been identified in breast tumor samples; one such mutation, Thr2609Pro, occurred specifically at the T2609 phosphorylation cluster (47). Taken together, these results suggest a tumor suppressor role of DNA-PKcs in the development of cancer.

In summary, this study focused on radiosensitivity and chromosomal instability in site-directed DNA-PKcs mutant cell lines after γ irradiation in the G₀/G₁ phase. Very clear differences in radiosensitivity were observed among cell lines mutated in the T2609 cluster, the S2056 cluster and the PI3K kinase domain. Additionally, an interesting synergism between defects in phosphorylation sites that occur together in both the S2056 and T2609 clusters was found to be critical for conferring maximum hypersensitivity to radiation-induced cell killing, comparable to DNA-PKcs null mutants.

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FIG. 1.

Summary of DNA-PKcs mutations within the S2056 cluster, T2609 cluster and PI3K kinase domain. I, S2056 phosphorylation cluster (refs. 16-18). II, T2609 phosphorylation cluster (refs. 19,20). III, PI3K domain (ref. 15).

FIG. 2.

Panel A: Survival curves of site-directed mutant cell lines with amino acid replacement in the S2056 and T2609 clusters. Colored areas indicate differential radiosensitivities: green, $D_0 = 1.0\text{--}1.4$ Gy; yellow, $D_0 = 0.6\text{--}0.9$ Gy; red, $D_0 = 0.35\text{--}0.7$ Gy. Panel B: Survival curves of site-directed mutant cell lines with replacement of amino acid or frame-shift mutation on PI3K domain. Colored areas indicate differential radiosensitivities as described above.

TABLE 1

Cell Lines Derived from DNA-PKcs Null V3 Cells with Complemented Human DNA-PKcs cDNA Containing Amino Acid Substitutions at Various Positions in the DNA-PKcs Constructs

Coded mutant cell lines	Altered DNA-PKcs mutants (complemented cDNA)	Substituted domains												
		S2056 cluster ^d					T2609 cluster ^d					PI3K ^{b,c,d,e}		
		S2023	S2029	S2041	S2051	S2056	T2609	S2612	T2620	S2624	T2638	T2647	Y3715	L3750 K3752 D3921
L-1	Wild-type													
L-2	V3-7A (S2056A +T2609 cluster to A)					A	A	A	A	A	A	A		
L-3	V3-6A (T2609 cluster to A)						A	A	A	A	A	A		
L-4	V3-2A (S2056A/T2609A)					A	A							
L-5	V3-S2056A					A								
L-6	V3-T2609A						A							
L-7	V3 (empty vector)													
L-8	V3-KA4 (kinase dead)												R	R
L-9	V3-KB20 (kinase dead)												R	R
L-10	V3-KC23 (kinase dead)											D		
L-11	V3-KD51 (kinase dead)													N
L-12	V3-5A (S2056 cluster to A)	A	A	A	A	A								
L-14	V3-3A (T2609A/T2638A/T2647A)					A	A			A	A	A		

^aSerine (S) and threonine (T) substituted by alanine (A) in the S2056 and T2609 cluster sites.

^bV3-KC23 mutant carries a frame-shift at position of amino acid 3715 that resulted in truncation of the protein after 10 amino acids and loss of the entire PI3K kinase domain.

^cV3-KA4 mutant substituted lysine 3752 (K3752) to arginine (R).

^dV3-KB20 mutant substituted leucine 3750 (L3750) and K3752 to arginine (R).

^eV3-KD51 mutant substituted aspartic acid (D) to asparagine (N).

TABLE 2

Characteristics of Nonirradiated Chinese Hamster Cell Lines

Cell line	Type of mutation	Percentage of S-phase cells ^a			MDT (h) ^b	Model chromosome no. per cell (%) ^c
		IL ⁻	Asyn			
CHO	Wild-type	3.4	61.5		14	21 (39.3)
AA8	Wild-type	1.4	54.5		13	20 (40.0)
Xrs5	Ku80 null	10.5	51.0		18	20 (32.0)
L-1	Wild-type	2.1	47.0		16	21 (19.0)
L-2	DNA-PKcs	5.6	43.4		15	34 (32.0)
L-3	DNA-PKcs	7.7	43.3		14	30 (33.0)
L-4	DNA-PKcs	5.2	51.2		15	35 (21.0)
L-5	DNA-PKcs	2.3	45.8		16	20 (19.6)
L-6	DNA-PKcs	12.2	49.8		17	20 (50.0)
L-7	DNA-PKcs null	0.8	59.8		18	20 (25.0)
L-8	DNA-PKcs	13.4	48.6		18	20 (41.0)
L-9	DNA-PKcs	13.1	52.2		14	33 (24.0)
L-10	DNA-PKcs	12.1	51.0		18	21 (52.0)
L-11	DNA-PKcs	2.4	50.6		16	20 (44.0)
L-12-10 ^d	DNA-PKcs	6.7	50.5		26	30 (18.0)
L-12-15 ^d	DNA-PKcs	2.0	48.2		16	33 (17.0)
L-14	DNA-PKcs	6.1	42.7		25	36 (18.0)

^aPercentage of cells incorporating BrdU during a 15-min pulse-labeling after 48 h IL-deficient synchronization or in asynchronous cell population.

^bMean doubling time (MDT) was determined from the linear portion of growth curves of the exponentially growing cell population.

^cModel chromosome number per cell. More than 100 metaphase cells were scored for each cell line. No significant differences in ploidy were found between nonirradiated and irradiated cells. Numbers in parentheses are percentages of cells scored with model chromosome number.

^dTwo clones were isolated separately as independent mutant clones.

TABLE 3
Gamma-Ray Sensitivity and Chromosomal Aberration Induced in DNA-PK Mutant Cell Lines

Cell line	Type of mutation	D_0 (Gy) ^a	D_{10} (Gy) ^b	Total chromosome aberrations per cell ^c		Radiation sensitivity ^d
				0 (Gy)	0.5 (Gy)	
CHO	Wild-type	1.17 ± 0.03	4.13 ± 0.19	0.019 (159)	0.057 (159)	N
AA8	Wild-type	1.35 ± 0.10	4.55 ± 0.19	0.038 (159)	0.094 (159)	N
Xrs5	Ku80 null	0.68 ± 0.04	0.72 ± 0.02	0.110 (106)	1.450 (106)	SSS
L-1	Wild-type	1.08 ± 0.05	3.50 ± 0.46	0.019 (53)	0.170 (53)	N
L-2	DNA-PKcs	0.37 ± 0.02	1.14 ± 0.05	0.153 (53)	0.271 (53)	SSS
L-3	DNA-PKcs	0.42 ± 0.03	1.12 ± 0.08	0.135 (159)	0.574 (53)	SSS
L-4	DNA-PKcs	0.47 ± 0.04	1.20 ± 0.07	0.128 (53)	0.275 (53)	SSS
L-5	DNA-PKcs	0.96 ± 0.06	2.87 ± 0.15	0.075 (53)	0.094 (53)	S
L-6	DNA-PKcs	0.92 ± 0.08	2.64 ± 0.14	0.075 (53)	0.171 (53)	S
L-7	DNA-PKcs null	0.36 ± 0.02	1.09 ± 0.04	0.094 (53)	0.660 (53)	SSS
L-8	DNA-PKcs	0.38 ± 0.03	1.00 ± 0.05	0.059 (53)	0.563 (30)	SSS
L-9	DNA-PKcs	0.39 ± 0.04	1.14 ± 0.20	0.039 (53)	0.270 (53)	SSS
L-10	DNA-PKcs	0.46 ± 0.02	1.21 ± 0.06	0.057 (106)	0.547 (106)	SSS
L-11	DNA-PKcs	0.36 ± 0.02	1.15 ± 0.05	0.094 (53)	0.226 (53)	SSS
L-12-10	DNA-PKcs	0.75 ± 0.06	2.25 ± 0.39	0.189 (53)	0.389 (53)	SS
L-12-15	DNA-PKcs	0.70 ± 0.12	2.11 ± 0.09	0.094 (106)	0.554 (106)	SS
L-14	DNA-PKcs	0.63 ± 0.02	1.50 ± 0.10	0.100 (53)	0.179 (53)	SS

^a D_0 , a parameter in the multitarget equation ($S = 1 - [1 - \exp(-DD_0)]^P$); the radiation dose that reduces survival to e^{-1} (i.e. 0.375) of its previous value on the exponential portion of the survival curve. Each point represents of the mean ± SEM of the results from more than three separate experiments.

^b D_{10} , radiation dose required to reduce survival to 10%.

^cFrequencies of chromosomal aberrations were normalized to 21 chromosome per cell based on model chromosome number per cell (Table 2). Numbers in parentheses are numbers of cells scored.

^dRadiosensitivity based on the D_0 value: N, normal (D_0 1.0–1.4 Gy); S, slightly sensitive (D_0 0.9 Gy); SS, sensitive (D_0 0.6–0.9 Gy); SSS, very sensitive (D_0 0.35–0.7 Gy).