Original paper

Comet assay is not useful to predict normal tissue response after radiochemotherapy in cervical and larynx cancer patients

Agnieszka Adamczyk[1], Beata Biesaga[1], Małgorzata Klimek[2], Anna Mucha-Małecka[3]

[1]Department of Tumour Pathology, Maria Sklodowska-Curie Memorial Cancer Centre and Institute of Oncology, Krakow Branch, Poland
[2]Gynecology Department, Maria Sklodowska-Curie Memorial Cancer Centre and Institute of Oncology, Krakow Branch, Poland
[3]The Oncology Unit Departament, Maria Sklodowska-Curie Memorial Cancer Centre and Institute of Oncology, Krakow Branch, Poland

Normal tissues reactions after radiotherapy vary considerably even between patients receiving the same treatment. The ability to predict the differences in radiosensitivity before radiotherapy would have important implication.
Patients with squamous cell carcinoma of the: (i) cervix (38 patients) and (ii) larynx (19 patients) were studied. Control group consisted of 9 healthy women. To assess individual radiosensitivity/chemoradiosensitivity alkaline version of comet assay was performed using isolated peripheral blood lymphocytes from cancer patients and healthy donors. The level of endogenous (0Gy), initial (immediately after 6Gy irradiation) and residual (after irradiation and 1h of repair) DNA damage was investigated. The mean value of endogenous damage was similar in control and cervical cancer (CCU) groups and significantly lower than in larynx cancer patients. Cancer patients showed slower DNA repair. For CCU and larynx patients, comet assay parameters were not helpful for unequivocal prediction of appearance of acute and late radiation reaction effects.
Comet assay seems to be unable to predict normal tissue reaction after radiochemotherapy. Therefore, there is still need for developing predictive assays, however, due to complicated mechanism of chemoradiosensitivity, only assays assessing not one but many molecular pathways might gives us reliable score.

Key words: comet assay, chemoradiosensitivity, cervix cancer patients, larynx cancer patients.

Introduction

Ionizing radiation is widely and successfully applied in oncology. However despite the advanced radiotherapy schedules, the success of radiotherapy still depends on the total radiation dose, which is limited by the tolerance of normal tissue surrounding tumor. Normal tissues reactions vary considerably even between patients who receive the same treatment. Several factors which can be patient and treatment – related are known to influence the variability of side effects. However, about 70 percent of variability cannot be explained by those circumstances. It is believed that individual differences in normal tissue damage are caused by variation in intrinsic radiosensitivity [1]. The ability to predict the differences in

radiation sensitivity would have important implication with regard to cancer treatment. This is why, there is much interest among clinicians for *in vitro* detection of cellular radiosensitivity which could reflect and foresee patients' normal tissue reaction after therapy [2, 3, 4, 5, 6, 7]. Currently most of the schedules include radio and chemotherapy, and there is need for simple test assessing individual sensitivity, which can be used in clinical practice. The use of such assays would enable clinicians to adjust schedules for both sensitive and resistant patients and also improve the therapeutic ratio. For many years great effort was made to identify assays, which can be used to predict the risk of the acute or late radiation reactions in single patient.

Assuming that intrinsic sensitivity is genetically determined, different cells (epidermal cells, fibroblasts, lymphocytes) from the same patient could be used to measure sensitivity to radiation or chemotherapeutic drug. The peripheral blood lymphocytes are one of the most widely used cells for *in vitro* radiosensitivity studies, because they can be easily obtained by venipuncture and they do not require complicated culture procedures. The assays which are estimating clonogenic survival and chromosomal/chromatin aberration are time consuming and not suitable for studies of a large number of patients. Assay which are based on electrophoresis are much quicker. In particular, the alkaline single cell microgel electrophoresis assay has been shown to be useful for assessment of DNA damage and repair [8]. This technique, also called comet assay, requires a small number of cells suspended in a thin agarose gel on a microscope slide, which are lysed, electrophoresed, and stained with a fluorescent DNA-binding dye. Damaged, fragmented DNA migrates out of the nucleus in the electric field towards the anode, which resembles the shape of a comet with a brightly fluorescent head (the nucleus) and a "tail" (fragmented DNA). The main advantage of comet assay is its ability to provide information regarding the damage and repair capacity of single cell. The comet assay is used in variety of different fields like genotoxicity testing, biomonitoring studies and clinical studies [9, 10, 11, 12]. Usefulness of comet assay is also investigated in regard of its ability to predict acute and late normal tissue damage after cancer treatment or differences between cancer patients and healthy donors [13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34]. Therefore, the aim of our study was to analyze the value of comet assay as predictor of normal tissue effect after radiochemotherapy in cervical and larynx cancer patients.

Material and methods

Study subjects

Thirty eight patients with carcinoma of the cervix and eighteen patients with squamous cell carcinoma of the larynx treated at Cancer Centre and Institute of Oncology, Cracow Branch were included into the study. None of the patients were earlier treated with chemotherapy or radiotherapy.

The group with carcinoma of the cervix consists of 19 with IIB and 19 with IIIB FIGO stage patients. The mean age was 56.6 year and ranged from 41-80 years. The treatment consisted of radiotherapy and chemotherapy. Radiotherapy was conducted in two phases: external beam irradiation and brachytherapy. External beam irradiation was given in 1.8 or 2 Gy per fraction to total dose from 44 to 60 Gy. All patients were given chemotherapy based on cisplatin (median: 5 cycles, 70 mg, mean: 4.4 cycles, 70 mg). The brachytherapy was performed using ^{137}Cs source with two (2 × 20 Gy to point A) or three (3 × 13,3 Gy to point A) series separated by one week interval.

In the group with carcinoma of the larynx the mean age was 56.7 year and ranged from 45-70 years. The treatment consisted of concurrent chemoradiotherapy. External beam irradiation was given in 2 Gy per fraction to total dose from 60 to 70 Gy. During radiotherapy all patients were given chemotherapy based on cisplatin (median: 3 cycles, 100 mg, mean: 2.9 cycles, 87 mg).

Control group consist of 9 healthy women. The healthy donors were selected to represent the similar age range as patients group (the mean age: 55.2 year, range: 42-79 years).

Acute radiation reactions were assessed using EORTC/RTOG scale and late radiation reaction were assessed after 6 and 12 months. Normal tissue reactions were assessed in bladder, bowel and anus for cervical patients and in skin, subcutaneous tissue, mucosa and salivary glands for larynx cancer patients. Additionally for larynx patients late radiation reactions were scored using SOMA scale. The study gained approval from the Ethical Committee at Maria Sklodowska-Curie Memorial Cancer Centre and Institute of Oncology in Krakow (decision from 17th September 2004). During radiotherapy, an acute radiation reaction occurs in patients, which can persist up to 6 months after the end of treatment. Its course is gradual (from grade 0, G0 – without any reaction to grade 4, G4 – most severe), and its severity depends on the individual sensitivity of healthy tissues to radiation, radiation dose and volume of irradiated tissue. During radiotherapy of the laryngeal cancer, inflammation of the skin and mucousa of the irradiated region, sore throat, and dry mouth can develop. Irradiation of the cervical cancer, causes abdominal

pain, nausea, vomiting, disturbances in urination and defecation, bloating, and bleeding from the lower part of the gastrointestinal tract. During irradiation, there is a need to monitor and treat acute radiation reaction because its high severity may need to stop treatment. Six months after the end of radiotherapy late radiation reactions may occur. Symptoms for late reaction are similar to acute radiation reaction, and scale used for classification ranges from G0 (luck of reaction) to G4 (severe reaction).

Blood collection, isolation of lymphocytes and irradiation *in vitro*

The experiment design is presented on Fig. 1. Blood samples from patients were obtained before treatment, during routine blood collection for diagnostic purposes. Blood from healthy donors and cancer patients was collected to tubes with heparin and lymphocytes were isolated using Histopaque 1077 (Sigma) according to manufacturer procedure. Then viability and concentration of cells were counted and cells were frozen and kept at –70°C.

One day before performing comet assay cell were thawed and cultured (RPMI 1640, 10% fetal calf serum, penicillin/streptomycin 100 IU/100 μg per ml). One hour before irradiation cisplatin to final concentration: 100 μM and 250 μM was added to dedicated test tubes containing cells from larynx cancer patients. Cells were irradiated on ice with X-rays at dose rate 2.6 Gy/min with dose 6 Gy. To estimate DNA repair, cells were kept at 37°C and 5% CO_2 for 60 or 120 min after 6 Gy irradiation.

Alkaline comet assay, acquisition and analysis of images

After irradiation cells were mixed with LMP agarose type VII (final concentration 1%), spread onto the slide precoated with 1% agarose type I-A and covered with coverslips. The slides were moved to 4°C to solidify and coverslips were removed. Then, slides were transferred to lysis buffer (pH 10) for 1 h at 4°C and later washed in TAE buffer and transferred to unwinding buffer for 20 min at 4°C. Electrophoresis was carried out at 0.8 V/cm for 25 min at 4°C. After electrophoresis the slides were washed three times in distillated water, once in ice cold 100% ethanol, dried at room temperature and store at 4°C. For visualization slides were stained with propidium iodine (Fig. 2). Fifty images were stored for each time point using microscope OLYMPUS BX-41 and

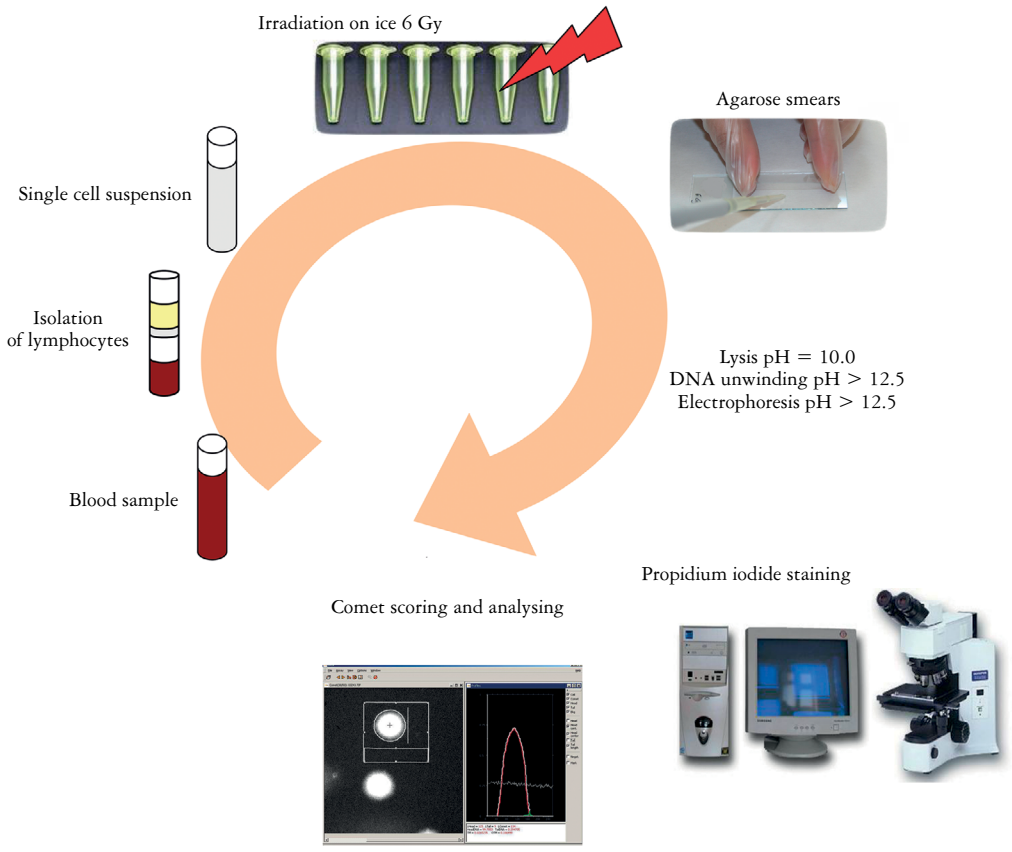

Fig. 1. Scheme of the experiment. Lysis buffer: 2,5 M NaCl, 100 mM $EDTANa_2$, 10mM TRIS, 1% sodium N-lauroyl sarcosinate, 10% dimethylsulfoxide, 1% Triton X-100, unwinding buffer: 0.1 M NaOH, 1 M NaCl, 1 mM $EDTANa_2$, electrophoresis buffer: 0.1 M NaOH, 1mM $EDTANa_2$

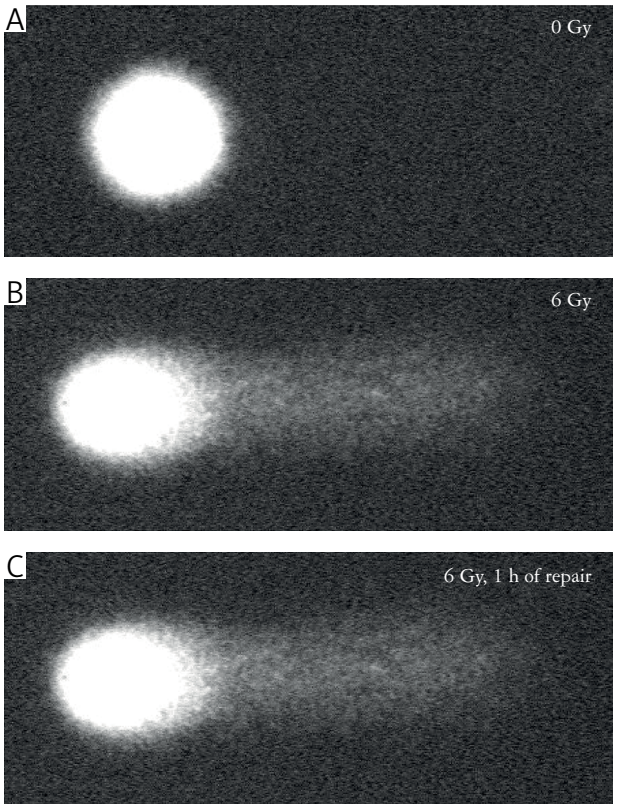

Fig. 2A-C. Examples of comet images: A) not irradiated, B) irradiated with dose of 6 Gy, clearly visible head (the nucleus) and a tail (fragmented DNA), C) irradiated with dose of 6 Gy and left for 1h to allow repair, visible small tail containing still fragmented DNA

MultiScan program (Computer Scanning Systems, Ltd.). The dead and apoptotic cells were excluded. The analysis of stored comets images were performed using CASP software. The program estimates several parameters of each comet, however "tail moment" (TM) was chosen to assess the level of DNA damage. The value of TM is defined as percentage of DNA in the comet tail multiplied by tail length.

Cisplatin causes DNA cross-linking at the cellular level, thus resulting in shorter DNA tail after irradiation. This process can be expressed as the percentage decrease in tail moment and was calculated for samples treated with cisplatin [35].

Statistics analysis

STATISTICA 10 software, (StatSoft, Inc., Tulsa, OK, USA) was used for calculations. Threshold for statistical significance was set at $p < 0.05$. Correlation for continuous variable was estimated using Pearson correlation. One-way ANOVA test was used with post-hoc Tuckey test to estimate differences between more that two groups. The Students test for dependant or independent variables was performed to measure differences between two groups.

Results

Comparison between groups

The level of endogenous (0Gy), initial (directly after irradiation with dose of 6Gy) and residual (after 1 h of repair) damage of DNA in cervical, larynx cancer and control groups are presented in the Table I. In all groups' inter-individual variation was observed for all parameters. There was no correlation between age of the patients and healthy women and any investigated comet assay data. The mean value of endogenous damage was similar in control and cervical cancer groups (Table I, Fig. 3). The significantly higher level of endogenous damage was detected in larynx patients group comparing to healthy donors and cervical cancer patients. There was difference in rate of DNA repair between patients and healthy donors. In the patients group, level of residual damage was significantly higher than level of endogenous damage. Also level of residual damage in larynx patients group was significantly higher than in cervical cancer group (Table I, Fig. 3).

Cisplatin effect

Cisplatin causes DNA cross-linking at the cellular level, thus resulting in shorter DNA tail after irradiation. The tail moment for cells incubated with

Table I. Endogenous, initial and residual values of tail moment measured in lymphocytes from cervical, larynx cancer and control group

	Tail moment (mean ±SE)		
	Endogenous 0 Gy mean ±SE	Initial 6 Gy mean ±SE	Residual 6 Gy, 1 h of repair mean ±SE
Cervical patients	0.050 ±0.07	64.48 ±2.50[a]	4.03 ±0.68[a]
Larynx patients	1.17 ±0.26	77.84 ±9.90[a]	10.00 ±1.81[a]
Healthy donors	0.34 ±0.09*	79.58 ±6.12[a]	0.94 ±0.33**

*One-way ANOVA - differences between groups: *p = 0.002 (post-hoc RIR Tuckey, healthy donors vs. CCU cancer patients: 0.004, CCU cancer vs. larynx cancer patients: 0.013), **p < 0.001 (post-hoc RIR Tuckey, healthy donors vs. larynx cancer patients: < 0.001, CCU patients vs. larynx cancer patients: 0.001)*

Student's test for dependent samples – differences between endogenous vs. initial and endogenous vs. residual damage: a = p < 0.001.

cisplatin and irradiated with dose of 6 Gy was significantly lower than for cells only irradiated (Table II). Also higher amount of cisplatin resulted in significantly lower value of tail moment. The value of decrease in tail moment for cell treated with 100μM of cisplatin was significantly lower than for cells treated with 250 μM of cisplatin (31.4% ±5.6 SE vs. 53.5% ±6.6 SE).

Damage caused by cisplatin was present even after one hour of repair (Table II). Values for tail moment after one hour of repair were significantly higher for cells incubated with cisplatin comparing with cells only irradiated (Table II).

Relation between comet assay parameters and acute and late radiation reaction

EORT/RTOG scale was used to assess acute and late normal tissue effects. Additionally for larynx patients late radiation reaction were also scored using SOMA scale (Table III). For analysis purpose, patients were divided into two groups: (i) patients without or with slight radiation reaction (G0 or G1) and (ii) patients with moderate and severe radiation reaction (higher than G1).

For CCU patients none of the comet assay parameters was helpful for prediction of appearance of acute and late (up to 6 months) radiation reaction. Patients who suffered from bladder and rectum ailment after 12 months had higher level of residual and endogenous damages respectively (Table IV).

Higher level of initial and residual damage (irradiation combined with cisplatin) was found in patients without acute skin and subcutaneous tissue reaction (Table V). Contrary, lower level of initial and residual damage (irradiation combined with cisplatin) was observed for patients without late (6 months) salivary gland reaction. Moreover, higher level of endogenous damage was observed for patients without salivary gland reaction (after 12 months). The slower repair (higher residual damage) was observed in patients experiencing late subcutaneous effects. The similar relationships were observed when SOMA scale was used to assess the late normal tissue effects (data not shown). Percentage decrease in tail moment after treatment with cisplatin was not related to any radiation reaction. Also, when patients were divided into two groups: without any tissue damage and with reaction in any tissue, we did not observe statistically significant differences in case of none of comet assay parameters.

Discussion

The alkaline comet assay was designed to assess DNA damages in single cell after application different harmful agents and to assess cell ability to repair those lesions. It also gives us baseline level of DNA damage, before exposure to agents.

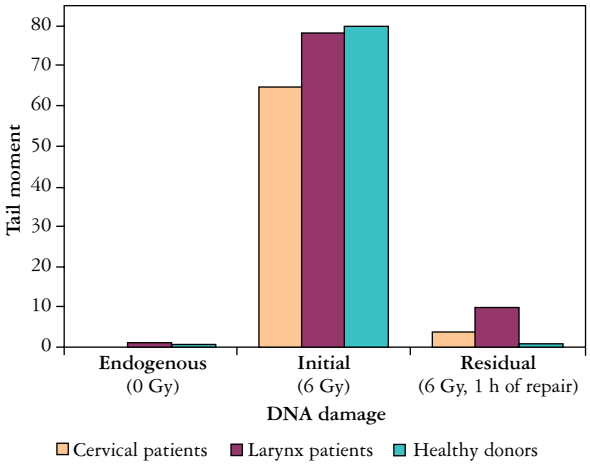

Fig. 3. Differences in level of endogenous, initial and residual DNA damage between cancer patients groups and healthy donors

Table II. Endogenous, initial and residual values of tail moment measured in lymphocytes from larynx cancer untreated or treated with cisplatin

	Tail moment mean ±SE
0Gy	1.17 ±0.26
6 Gy	77.84 ±9.90[a,]
6 Gy 1 h of repair	10.00 ±1.81[a, b]
6 Gy 100 μM CisPt	60.91 ±9.67[a, b, d,]
6 Gy 250 μM CisPt	41.76 ±8.33[a, b, d, e]
6 Gy 100 μM CisPt, 1 h of repair	65.90 ±10.25[a, c, d, f, h]
6 Gy 250 μM CisPt, 1 h of repair	54.19 ±9.81[a, b, d, e, g, h]

CisPt – cisplatin, Student's test for dependent samples:

[a] *difference between 0 Gy and other groups* $p < 0.001$

[b] *difference between 6 Gy and other groups* $p < 0.001$

[c] *difference between 6 Gy and 6 Gy 100 μM CisPt, 1 h of repair* $p = 0.002$

[d] *difference between 6 Gy 1 h repair and other groups* $p < 0.001$

[e] *difference between 6 Gy 100 μM and 6 Gy 250 μM* $p < 0.001$

[f] *difference between 6 Gy 100 μM and 6 Gy 100 μM CisPt, 1 h of repair* $p = 0.005$

[g] *difference between 6 Gy 100 μM and 6 Gy 250 μM CisPt, 1 h of repair* $p = 0.013$

[h] *difference between 6 Gy 250 μM and other groups* $p \leq 0.001$

In our study we observed elevated level for endogenous damage for larynx cancer patients comparing to control. Similar observation was reported by Polyvoda *et al*. in head and neck cancer patients [22]. Some authors observed higher level of endogenous damages in cervical cancer patients [14] and other types of cancer [17, 18, 20, 29, 30, 31] than in healthy donors. However lack of such differences was also reported (Table VI) [34]. In our study lymphocytes from cancer patients were characterized by slower repair comparing to healthy donors. This find-

Table III. Presence and intensity of acute and late radiation reactions in group of cervical and head and neck cancer patients

		CCU PATIENTS			LARYNX PATIENTS							
									SOMA			
		Bl	R	Bo	S	St	M	Sg	S	St	M	Sg
Accutey	G0	31	35	34				2				
	G1		1		10	8	1	4				
	G2	5	1	2	9	10	7	9				
	G3	1		1		1	11	4				
N			37				19					
After 6 month	G0	24	27	30				2				2
	G1	1			17	14	14	11	16	10	14	11
	G2	5	3	1		3	3	3	1	7	3	3
	G3							1				1
	G4											2
N			30				17				17	
After 12 month	G0	22	12	27	1			1	1			1
	G1	1	1		13	13	14	4	13	8	14	4
	G2	4	8			1		7		6		7
	G3		3					2				2
	G4		3					1				1
N			27				14				14	

Bl – bladder; R – rectum; Bo – bowel; S – skin; St – subcutaneous tissue; M – mucosa; Sg – salivary gland.

ing is confirmed by others studies concerning different types of cancer [17, 19, 22, 29, 30, 31, 33].

Our data concerning usefulness of comet assay for prediction of normal tissue effects are not conclusive. In case of cervical cancer patients appearance of tissue injury after 12 months was significantly related to elevated level of endogenous damage in rectum and less efficient repair in bladder. In case of larynx cancer patients mostly initial and residual damage after irradiation and administration of cisplatin were relevant. However, these data also are ambiguous. Lower level of initial and residual damage was related to presence of skin and subcutaneous tissue damage but higher level of initial and residual damage was related to salivary gland damage. Moreover, literature data also are not consistent. Some authors reported lack of relationship between radiosensitivity assessed by comet assay and normal tissue damage after therapy [13, 15, 21] but others found association between tissue damage and impaired DNA repair [14, 23, 24, 26, 29, 28, 31] (Table VI). However, it is worth to mention that information provided by comet assay concerning repair, refer only to rate of DNA repair but not to fidelity of this process.

Data obtained by comet assay are very difficult to compare. Authors collected publication concerning usefulness of comet assay for assessment of intrinsic sensitivity of cancer patients, and presented them in Table VI. This approach will facilitate the demonstration of many problems that can be encountered by comparing the data from the comet test. One of them is scoring of DNA damage. There are different methods used, such as: tail moment [13, 19, 23, 28, 34], percentage of DNA in the tail [14, 17, 25, 27, 31], tail length [18, 33] or scoring of comets according to established types [16, 20, 22, 29]. Another problem is dose used for cells irradiation (1-35 Gy) and repair time (15 min – 24 h), which vary considerably between studies. So some discrepancies between published data can be caused by methodological differences what implies need for standardization [10].

Other problem emerges from Padjas *et al.* study [15], where three different tests: comet assay, G_0 (chromosome aberration) and G_2 (chromatid breaks) assays were used, all assessing DNA damage in single cell. Unfortunately, there was no correlation between those tests and each of them identified different patients as potentially radiosensitive. All those test measure effect of damaging agent on DNA of isolated, cultured cells. Although, it is considered that cells sensitivity should be regarded as complex process, which involves environmental factors as well as susceptibility alleles of large numbers of genes [3, 4, 5]. Model presented by Andreasen *et al*. [4, 5] might explain why, it is so difficult to find relation-

Table IV. DNA damage measured by comet assay stratified according to presence and severity of normal tissue reaction in cervical cancer group

	Acute radiation reaction mean ±SE							
	bladder		rectum		bowel		all	
	without reaction	with reaction	without reaction	with reaction	without reaction	with reaction	without reaction	with reaction
0 Gy	0.48 ±0.08	0.47 ±0.22	0.49 ±0.08	0.00	0.46 ±0.07	0.67 ±0.43	0.46 ±0.08	0.54 ±0.19
6 Gy	64.54 ±2.77	61.61 ±6.84	64.86 ±2.47	35.50	63.91 ±2.76	65.85 ±1.54	64.41 ±3.06	63.02 ±4.42
6 Gy 1 h repair	3.86 ±0.75	3.19 ±0.83	3.73 ±0.66	4.40	3.91 ±0.70	2.01 ±0.74	4.06 ±0.82	2.80 ±0.63
Late radiation reaction (6 months)								
0 Gy	0.44 ±0.08	0.58 ±0.34	0.43 ±0.08	0.80 ±0.40	0.47 ±0.08	0.20	0.40 ±0.07	0.61 ±0.22
6 Gy	61.41 ±3.30	67.86 ±5.71	61.54 ±3.04	70.97 ±10.54	62.40 ±3.01	64.90	59.88 ±3.64	68.57 ±4.33
6 Gy 1 h repair	4.33 ±0.90	2.97 ±1.23	4.184 ±0.85	3.37 ±1.70	4.03 ±0.80	6.20	4.38 ±1.05	3.46 ±0.88
Late radiation reaction (12 months)								
0 Gy	0.52 ±0.10	0.38 ±0.21	0.26 ±0.06	0.71 ±0.14[b]	0.50 ±0.14		0.29 ±0.07	0.64 ±0.13[c]
6 Gy	63.80 ±2.90	61.18 ±13.94	59.88 ±3.90	66.69 ±4.56	63.41 ±4.58		62.39 ±4.11	64.12 ±4.92
6 Gy 1 h repair	3.42 ±0.89	8.78 ±3.59[a]	2.90 ±0.53	5.43 ±1.49	4.21 ±1.49		2.69 ±0.56	5.26 ±1.31

Student's test for independent samples, [a]p = 0.021, [b]p = 0.004, [c]p = 0.010.

ships between results of *in vitro* tests, which assessed cell intrinsic radiosensitivity, with observed *in vivo* normal tissue reaction. Some genes may be expressed in all types of tissues but some are tissue specific. Also some test could measures influence of particular sets of genes on chemoradiosensitivity, which can be express differently in different types of tissues or cells. It is worth mention that *in vitro* environment can also influence expression of some genes and in that way diminishes predictive value of used tests [4, 5]. Question arises, if tests based on assessment of *in vitro* radiosensitivity of isolated cells, can reflect what is happening during irradiation on tissue level. *In vitro* tests do not take in to account involvement of cytokine-mediated interaction between different types of cells after interaction with damaging agents. Due to advancement in technology, new methods are used to find predictive markers for normal tissue reaction [2, 3, 36]. There are two ways to investigate possible causes of radiosensitivity: candidate gene approach and the genome-wide association study (GWAS) [37, 38]. Single nucleotide polymorphism (SNP) association studies using candidate gene approach usually looked for useful SNPs in genes coding DNA repair proteins, antioxidant enzymes and cytokines [37, 38] and there are arguments against this approach [39, 40]. However now, due to high-through genotyping, is possible to genotype even more then million SNPs in GWAS [6, 36, 37]. In genome wide association study one of the most important factor is large number of patients included in analysis, so conducting large studies is necessary [37, 40]. Due to complex nature of radiation induced injury, radiogenomic studies face unique difficulties in collecting and analyzing of data: (i) possible overlapping symptoms from radiation and from cancer development, (ii) variability in radiation protocols, (iii) "centre effect" and (iv) various system of scoring adverse effects [40]. It is worth to mention that in 2009 the Radiogenomics Consortium was funded, which members collaborate on studies to identify SNPs related to radiation induced adverse effects [37, 38]. GWAS conducted until now identify some SNPs reaching or approaching the genome wide significance level. However, those SNPs are not present in genes related to "radiobiology" meant in strict sense. Rather, these genes are involved in wide area of other physiological mechanisms: muscle regeneration,

Table V. DNA damage measured by comet assay stratified according to presence and severity of normal tissue reaction in larynx cancer group

	TAIL MOMENT MEAN ±SE							
	SKIN		SUBCUTANEOUS TISSUE		MUCOSA		SALIVARY GLAND	
	WITHOUT REACTION	WITH REACTION	WITHOUT REACTION	WITH REACTION	WITHOUT REACTION	WITH REACTION	WITHOUT REACTION	WITH REACTION
Acute radiation reaction								
0 Gy	1.17 ±0.14	1.18 ±0.43	1.30 ±0.37	1.08 ±0.36	1.71	1.14 ±0.27	1.47 ±0.58	1.04 ±0.27
6 Gy	95.48 ±13.07	58.24 ±12.70	100.43 ±14.49	61.41 ±11.57[d]	97.21	76.76 ±10.41	60.39 ±22.50	85.89 ±10.03
6 Gy 1 h of repair	11.04 ±2.50	8.85 ±2.74	11.56 ±3.07	8.87 ±2.27	4.56	10.30 ±1.89	9.98 ±3.00	10.01 ±2.35
6 Gy 100 μM CisPt	80.34 ±14.12	39.32 ±9.17[a]	85.21 ±17.05	43.24 ±8.28[e]	70.55	60.38 ±10.21	43.83 ±19.30	68.80 ±10.82
6 Gy 250 μM CisPt	54.92 ±12.72	27.13 ±8.75	64.35 ±13.91	25.32 ±7.30[f]	64.78	40.48 ±8.70	33.79 ±17.31	45.44 ±9.54
6 Gy 100 μM CisPt 1 h of repair	86.12 ±14.54	43.44 ±10.71[b]	92.06 ±17.54	46.88 ±9.16[g]	91.26	64.50 ±10.73	47.10 ±20.32	74.58 ±11.45
6 Gy 250 μM CisPt 1 h of repair	72.55 ±14.51	33.79 ±9.72[c]	81.15 ±16.67	34.57 ±8.14[h]	82.77	52.60 ±10.23	39.92 ±18.63	60.77 ±11.52
Late radiation reaction (6 months)								
0 Gy	1.13 ±0.26		1.03 ±0.29	1.64 ±0.63	1.169 ±0.31	0.97 ±0.45	1.24 ±0.33	0.79 ±0.34
6 Gy	78.93 ±11.05		74.54 ±12.29	99.45 ±26.33	71.12 ±11.92	115.39 ±20.46	68.43 ±12.13	113.07 ±18.57
6 Gy 1 h of repair	10.85 ±1.93		9.05 ±1.81	19.25 ±5.12[i]	10.46 ±2.24	12.66 ±3.83	10.01 ±2.07	13.58 ±5.03
6 Gy 100 μM CisPt	62.59 ±10.76		58.83 ±11.40	80.12 ±33.54	54.75 ±11.24	99.19 ±24.68	49.84 ±10.82	104.04 ±18.83[j]
6 Gy 250 μM CisPt	45.13 ±8.97		43.02 ±8.96	54.97 ±33.97	39.27 ±9.34	72.47 ±23.39	34.22 ±8.60	80.57 ±17.74[k]
6 Gy 100 μM CisPt, 1 h of repair	67.16 ±11.45		63.20 ±12.35	85.61 ±33.30	58.41 ±12.08	107.97 ±22.39	53.92 ±11.73	110.17 ±19.36[l]
6 Gy 250 μM CisPt, 1 h of repair	57.57 ±10.67		54.78 ±11.04	70.58 ±36.98	49.26 ±11.13	96.33 ±22.20	44.84 ±10.70	98.92 ±18.70[m]
Late radiation reaction (12 months)								
0 Gy	1.15 ±0.31		0.99 ±0.28	3.34	1.15 ±0.31		2.23 ±0.58	0.56 ±0.16[n]
6 Gy	83.48 ±10.06		84.05 ±10.85	76.09	83.48 ±10.06		89.64 ±22.76	80.05 ±10.39
6 Gy 1 h of repair	10.73 ±2.19		10.93 ±2.36	8.14	10.73 ±2.19		10.28 ±2.47	10.99 ±3.23
6 Gy 100 μM CisPt	64.78 ±9.90		66.27 ±10.57	45.42	64.78 ±9.90		60.70 ±21.08	67.04 ±11.06
6 Gy 250 μM CisPt	45.94 ±8.37		47.96 ±8.77	19.68	45.94 ±8.37		34.50 ±15.48	52.30 ±9.82
6 Gy 100 μM CisPt, 1 h of repair	69.90 ±10.79		71.74 ±11.48	46.00	69.90 ±10.79		64.46 ±22.04	72.92 ±12.48
6 Gy 250 μM CisPt, 1 h of repair	59.19 ±10.10		61.79 ±10.54	25.40	59.19 ±10.10		48.63 ±20.14	65.05 ±11.54

Student's test for independent samples, [a]p = 0.030, [b]p = 0.033, [c]p = 0.045, [d]p = 0.048, [e]p = 0.027, [f]p = 0.016, [g]p = 0.025, [h]p = 0.014, [i]p = 0.039, [j]p = 0.027, [k]p = 0.023, [l]p = 0.032, [m]p=0.026, [n]p=0.004.

Table VI. Revision of some published papers concerning use of comet assay for prediction of *in vivo* radiosensitivity and differences between healthy donors and cancer patients

Author [reference no]	Cancer/material	Comet assay version	Measurements	Statistical significance
Wang *et al*. [13]	Nosopharyngeal cancer/ lymphocytes	Alkaline	Tail moment: endogenous, initial (2.5, 5, 10 Gy), residual (after 15 and 30 min of repair)	No correlation between acute skin reaction and radiosensitivity assessed *in vitro*
Gabelowa *et al*. [14]	Cervical cancer/ lymphocytes	Alkaline	Percentage of tail DNA, exogenous, initial (1, 2, 3, 4 Gy) and residual (2 h)	endogenous damage for patients significantly higher than for healthy donors Acute radiation toxicity- patients with G0-G1 faster DNA strand break rejoing. Recurence associated with lower initial DNA damage (2 Gy) and increased level of DNA repair kinetics
Padjas *et al*. [15]	Breast and gynaecological cancer/lymphocytes	Alkaline	area under repair kinetic curve, (2 Gy, repair: 20, 40, 60, 80, 10, 120 min)	No correlation between early and late complications and assay's results
Cortes-Gutierrez *et al*. [16]	Cervical neoplasia/cervival epithelial cells	Alkaline and neutral	Score 0-3 type of comet, only exogenous	Increased level of endogenous dsb in patients with high grade squamous interepithelial leassons
Buchynska *et al*. [17]	Endometrial cancer/ Peripheral blood lymphocytes (PBL)	Alkaline	Percentage of DNA in tail, endogenous, initial after bleomycin-induced DNA damage, residual (15 min)	Increased level of endogenous and less effective DNA repair in patients compared to healthy control group
Kopjar *et al*. [18]	Breast cancer/ PBL	Alkaline	Tail length, endogenous	Increased level of endogenous damage in patients compared to healthy control group
Rajaee-Behbahani *et al*. [19]	Non-small cell lung cancer/PBL	Alkaline	Tail moment, endogenous, initial after bleomycin-induced DNA damage and residual (15 min)	Higher initial damage and less effective DNA repair in patients compared to healthy control group
Nadin *et al*. [20]	Various cancers/ PBL	Alkaline	Score 0-5 type of comet, exogenous initial (doxorubicin or cisplatin) and residual (24 h)	Increased level of endogenous damage in patients compared to healthy control group, decreased rate of cisplatin adducts repair in patients' PBL comparing to control
Twardella *et al*. [21]	Breast cancer/PBL	Alkaline	Tail moment endogenous, initial (5 Gy) and residual (30 min)	Parameters not associated with skin toxicity
Polyvoda *et al*. [22]	Head and neck cancer/ PBL	Alkaline	Score 0-4 type of comet endogenous, initial (2 Gy) and residual (15, 30, 60, 120, 180 min)	Increased level of endogenous damage in patients compared to healthy control group, in patients group more individuals with high DNA damage, low repair rate and high non-repaired DNA damage level

Table VI. Cont.

Author [reference no]	Cancer/material	Comet assay version	Measurements	Statistical significance
Popanda *et al*. [23]	Breast cancer/PBL	Alkaline	Tail moment endogenous, initial (5 Gy) and residual (15, 30 min)	No apparent correlation between acute skin reaction and endogenous damage, reduced repair capacity corresponds to very limited extend to acute skin radiosensitivity
Alapetite *et al*. [24]	Breast cancer and Hodkin's disease/ lymphocytes	Alkaline	Mean tail moment endogenous, initial (2, 5 Gy) and % of residual damage (15, 30, 60 min)	Increased level of residual damage after 30 and 60 min in the group of breast cancer patients with elevated acute and late skin reaction
Rusin *et al*. [25]	Head and neck cancer/ squamous cancer cells or healthy squamous cells isolated from tissue biopsies, PBL from healthy donors	Alkaline	Percentage of DNA in tail after gamma radiation and after gamma radiation and endonuclease treatment endogenous, initial (5, 15, 25, 35 Gy) and residual (30, 60, 120 min)	Cancer cells were more sensitive to genotoxic treatment and shoved impaired repair
Muller *et al*. [26]	Various tumours/PBL	Neutral	Ratio of DNA intensity in the tail to the head, background, initial (0.25, 0.5, 1, 2, Gy) and residual (15, 30, 60, 120, 180 min after 2 Gy)	Higher residual damage after 3h of repair in patients with severe side effects
Oppitz *et al*. [27]	Various tumours/skin fibroblasts, 4 ataxia telangiectasia cell lines		Percentage of DNA in tail endogenous, initial (1-15 Gy, 5 Gy chosen) and residual (10, 20, 30, 40, 50 min after 5 Gy)	Different kinetics of DNA repair (reduced capacity) between patients with acute/late tissue reaction and without reaction after 10, 20 , 30, 40 min, ataxia telangiectasia cell lines showed reduced repair at all time points comparing to both patients groups
Oppitz *et al*. [28]	Breast cancer/fibroblasts and lymphocytes	Alkaline	Tail moment endogenous (0 Gy), initial (3 Gy lymphocytes and 5 Gy fibroblasts) residual (5, 10, 20, 30, 40 min)	Significantly longer repair time only in lymphocytes from patients with elevated acute skin reaction comparing to average reacting patients
Shahidi *et al*. [29]	Breast cancer/lymphocytes	Alkaline and neutral	Score 0-4 type of comet and Olive tail moment endogenous, initial (0.25-16 Gy) and residual (1, 3, 24 h after irradiation with 4 Gy for alkaline and 8 Gy for neutral vesion)	Higher background of DNA damage for cancer patients than healthy donors. Less effective repair in the patients group than in controls
Smith *et al*. [30]	Breast cancer/lymphocytes	Alkaline	Comet tail moment endogenous (0 Gy), initial (6 Gy) residual (10 min)	Higher background, initial and residual damage in group of patients comparing to control

Table VI. Cont.

Author [reference no]	Cancer/material	Comet assay version	Measurements	Statistical significance
Sterpone *et al.* [31]	Breast cancer/ lymphocytes	Alkaline	Percentage of DNA in tail endogenous (0 Gy), initial (2 Gy) residual (30, 60 min)	Higher background, initial and residual damage in group of patients comparing to control. Less effective repair in the patients group with high degrees of tissue side effects
Rzeszowska-Wolny *et al.* [32]	Head and neck cancer/ lymphocytes	Alkaline	Visual classification endogenous (0 Gy), initial (2 Gy) residual (15, 30, 60, 120, 180 min)	Weak correlation between rate of DNA break repair and cumulative radiation dose causing the maximum acute reaction scored
Nascimento *et al.* [33]	Breast cancer/PBL	Alkaline	Comet length and visual classification (0-4 classes of comets) endogenous (0 Gy), initial (0.2-4 Gy) residual (3, 24 h)	Slower repair in cancer patients comparing to healthy donors
Djuzenowa *et al.* [34]	Breast cancer/peripheral blood mononuclear cells	Alkaline	Tail moment endogenous (0 Gy), initial (5 Gy) residual (10, 20, 30, 40 min)	No differences between cancer patients and healthy donor, cancer patients with adverse early skin reaction and healthy donor group in endogenous, initial, residual DNA damage level as well as in DNA repair kinetics

hormone metabolism, inflammation, ion-channeled muscle contraction [37].

Concluding, our results of alkaline comet assay corresponded only at very limited extend to occurrence of acute and late normal tissue reaction after irradiation in CCU and larynx cancer patient. Taking into account contradicting data concerning usefulness of comet assay to predict *in vivo* normal tissue damage, it seems that comet assay is not suitable to select chemoradiosensitive patients, who will suffer from moderate or severe damage of normal tissue after radiation. Although, the comet assay is used with success in genotoxicity testing and biomonitoring studies, this test is not useful for selection of chemoradiosensitive patients. Therefore, there is still need for identifying predictive assays concerning normal tissue chemoradiosensitivity. However, due to complicated mechanism of this process, only assays assessing not one but many molecular pathways might gives us reliable score.

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Address for correspondence

Agnieszka Adamczyk
Department of Tumour Pathology
Maria Sklodowska-Curie Memorial Cancer Centre
and Institute of Oncology, Krakow Branch
Garncarska 11
31-115 Krakow, Poland
tel.: +48 12 63 48 415
fax: +48 12 423 1076
e-mail: z5adamcz@cyfronet.pl