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Searching for *in vitro* biomarkers of susceptibility to prostate and cervical cancers by analysis of chromosomal instability, γ-H2AX foci, polymorphisms in DNA repair genes and apoptosis

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Abstract

Introduction and objective. According to the cancer epidemiology databases, cancer is the second leading cause of death in developing countries. Moreover, the WHO predicts a continuing increase in the incidence of cancer, extending this trend well into the next several decades. Hence, it seems obvious that the prediction of cancer susceptibility and early diagnosis is an important goal for modern biomedical sciences. The aim of this study is to clarify the value of chromosomal damage, capacity for the repair of double-strand breaks (DSBs), polymorphisms in DNA repair genes, and apoptosis as prognostic markers for prostate and cervical cancer.
Materials and methods. 30 prostate cancer patients and 30 cervical cancer patients were enrolled into the study. In addition, 30 healthy female donors and 30 healthy male donors served as controls. The following endpoints were investigated: frequency of micronuclei, gamma-H2AX fluorescence, *XRCC1* 194C>T, *XRCC1* 399G>A, XRCC3 IVS5–14 A>G, OGG1 326 Ser>Cys polymorphisms and apoptosis.
Results. Among all tested factors, only the homozygous variant (Arg/Arg) in XRCC1 (399 Arg/Gln) was strongly associated with prostate cancer risk, and only a low apoptotic response was connected with cervical cancer risk. The presented study confirmed a positive association between the frequency of MN and increased prostate and cervical cancer risk. However, such a biomarker is not cancer specific. In addition, the information gained by analyzing the gamma-H2AX fluorescence, as well apoptosis, had no value for predicting the risk of prostate and cervical cancers.
Conclusions. The final conclusion of the study is that cancer susceptibility is a complex phenotype not readily detectable in relatively small studies by functional assays or analysis of SNP in few, selected genes.

Key words

cervical cancer patients, prostate cancer patients, healthy donors, human lymphocytes, chromosomal instability, γ-H2AX foci, apoptosis, SNP polymorphisms in XRCC1, XRCC3 and OGG1 genes, ionizing radiation.

INTRODUCTION

According to the cancer epidemiology databases provided by the International Agency for Research on Cancer and the WHO Cancer Mortality Database, cancer is the second leading cause of death in developing countries. Moreover, the WHO predicts a continuing increase in the incidence of cancer, extending this trend well into the next several decades. Hence, it seems obvious that the prediction of cancer susceptibility and early diagnosis is an important goal for modern biomedical sciences. To-date, a number of various biomarkers of cancer predisposition have been studied, but despite the great expansion of knowledge over the past several decades and the development of technology, existing data on sensitive and specific biomarkers are still scarce and contradictory. The results of several single studies show that an enhanced frequency of spontaneous chromosomal aberrations in peripheral blood lymphocytes of healthy individuals may be associated with an enhanced risk of developing cancer, as reviewed in Hagmar [1]. These data have been supported by the results of epidemiological studies,

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including 11 national cohorts and a total of 22,358 cancer-free individuals [2]. However, it seems that not only a high level of spontaneous cytogenetic damage, but also increased sensitivity to mutagens, correlate with the increased risk of cancer [3]. Many researchers who used ionizing radiation as a mutagenic agent reported that chromosomal radiosensitivity was higher in the lymphocytes of cancer patients than in healthy donors [4]. Other studies indicate that the increased susceptibility to cancer is primarily associated with the efficacy of DNA repair, and this may be affected by variations in several genes, including DNA repair genes [5]. In addition, the potential association of susceptibility to apoptosis with cancer risk has been investigated. However, all available results are not always consistent with one another, partially because of the small sample size of some published studies, different ethnic backgrounds, or different types of cancer [6].

The presented study focuses on the role of chromosomal damage, the capacity for the repair of double-strand breaks (DSBs, polymorphisms in DNA repair genes, and apoptosis in the prediction of prostate cancer, the second most common cancer among men, and cervical cancer, the third most common malignancy among women [7]. To obtain comprehensive results, both spontaneous and radiation-induced response was investigated in the lymphocytes of cancer patients and healthy donors. The analyzed endpoints were the frequency of micronuclei, frequency of apoptotic/necrotic cells and intensity of gamma-H2AX fluorescence. In addition, single nucleotide polymorphisms (SNPs) in *XRCC1* 194C>T, *XRCC1* 399G>A, *XRCC3* IVS5–14 A>G, *OGG1* 326 Ser>Cys genes were determined, that encode proteins involved in different repair pathways and that have been investigated in published epidemiological studies of cancer.

MATERIALS AND METHOD

The study was approved by the Ethics Committee of the Maria Sklodowska-Curie Memorial Cancer Centre and Institute in Warsaw, Poland (Ref. No. 6/2007). All peripheral blood samples were collected with the written consent of the patients and healthy donors.

All cancer patients were admitted to the Oncology Centre in Kielce, Poland, between May 2007 – October 2011. 30 prostate cancer patients (PP), average age: 66.6±7.9, and 30 cervical cancer patients (CP), average age: 57.6±10.3, were enrolled. All diagnoses were verified by histological examination of the tumours and clinical data, such as FIGO or TNM classification (Tab. 1). In addition, 30 healthy female donors (HF), average age: 57.8±10.4, and 30 healthy male donors (HM), average age: 55.6±8.6, served as controls. The age distribution of all donors is shown in Table 1.

Blood sample processing. Human venous blood samples were taken from all patients before the initial treatment and from healthy donors, collected into heparinized (10–20 U/ml) Greiner bio-one tubes (Greiner Bio-One GmbH, Frickenhausen, Germany), and divided between 2 test tubes: one to determine the spontaneous level of biological endpoints and the other to be irradiated *in vitro* with 2 Gy (^{60}Co, dose rate 1.13 Gy/min, 200 kV, Siemens Theratron Elite 80, Forchheim, Germany, at room temperature). Blood samples were then processed for further analyses.

Table 1. Details on cervical and prostate cancer patients and healthy donors

Cervical cancer patients			Prostate cancer patients			Healthy female DONORS		HEALTHY MALE DONORS	
Code	age	FIGO Classification	CODE	age	TNM Classification	L.P.	age	L.P.	AGE
CP1	54	III	PP1	64	T3N0M0 Gleason	HF1	54	HM1	52
CP2	63	IC	PP2	81	T3NXM0 Gleason	HF2	56	HM2	42
CP3	67	IIB	PP3	46	T3NXM0 Gleason	HF3	56	HM3	56
CP4	76	IIB	PP4	61	T3NXM0 Gleason 5	HF4	64	HM4	59
CP5	55	III	PP5	53	T2NXM0 Gleason 7	HF5	73	HM5	63
CP6	68	IC	PP6	74	T2N0M0 Gleason 6	HF6	70	HM6	52
CP7	64	IC	PP7	69	T2N0M0 Gleason 6	HF7	34	HM7	63
CP8	43	IIB	PP8	66	T3N0M0 Gleason 6	HF8	78	HM8	64
CP9	71	IIB	PP9	69	T3NXM0 Gleason 7	HF9	62	HM9	48
CP10	72	IC	PP10	63	T3NXM0 Gleason 7	HF10	58	HM10	60
CP11	64	IC	PP11	49	T3NXM0 Gleason 7	HF11	53	HM11	62
CP12	54	I	PP12	65	T3NXM0 Gleason 9	HF12	55	HM12	64
CP13	44	III	PP13	69	T2N0M0 Gleason 7	HF13	56	HM13	43
CP14	56	I	PP14	66	T3N0M0 Gleason 7	HF14	59	HM14	58
CP15	53	I	PP15	71	T3N0M0 Gleason 6	HF15	60	HM15	39
CP16	76	II	PP16	70	T3N0M0 Gleason 7	HF16	70	HM16	57
CP17	53	I	PP17	74	T3N0M0 Gleason 9	HF17	67	HM17	52
CP18	54	I	PP18	67	T3N0M0 Gleason 7	HF18	46	HM18	52
CP19	54	III	PP19	77	T3N0M0 Gleason 7	HF19	61	HM19	44
CP20	53	II	PP20	70	T3N0M0 Gleason 7	HF20	53	HM20	50
CP21	59	I	PP21	53	T3N0M0 Gleason 7	HF21	67	HM21	48
CP22	47	IIB	PP22	66	T3N0M0 Gleason 6	HF22	69	HM22	67
CP23	48	III B	PP23	74	T2NXM0 Gleason 6	HF23	60	HM23	61
CP24	53	I	PP24	67	T1N0M0	HF24	68	HM24	60
CP25	74	I	PP25	72	T2NXM0	HF25	49	HM25	41
CP26	43	III	PP26	73	T3N0M0 Gleason 9	HF26	61	HM26	72
CP27	43	I	PP27	74	T3NXM0	HF27	45	HM27	64
CP28	54	III	PP28	61	T2N0M0 Gleason 6	HF28	37	HM28	52
CP29	46	I	PP29	67	T3N0M0 Gleason 6	HF29	43	HM29	60
CP30	69	II	PP30	67	T3N0M0 Gleason 6	HF30	52	HM30	64

Determination by micronucleus assay of chromosomal damage. Cultures of lymphocytes were prepared by adding 0.5 mL of whole blood to 4.5 mL of RPMI 1640 medium supplemented with 20% foetal bovine serum, 10 µg/ml PHA, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cultures were incubated at 37 °C for 68 h. Cytochalasin B (final concentration of 10 µg/mL) was added after 44 h of incubation in order to block cytokinesis and obtain binucleated cells. After an additional 24 h incubation at 37 °C, the cells were harvested by centrifugation and subjected to mild hypotonic treatment (0.14 M KCl) for 5 min., fixed twice with 0.9% NaCl, methanol and acetic acid (13:12:3) and then smeared onto pre-cleaned microscope slides and air dried. The slides were stained with 5% Giemsa diluted in phosphate buffer (0.06 M Na_2HPO_4 and 0.06M KH_2PO_4, pH 6.8) for 5 min, washed with distilled water, air-dried and kept until microscopic analysis. The frequency of micronuclei (MN) was determined by a blind test in 1,000 binucleated cells with the cytoplasm well preserved using a Nikon Eclipse 400 microscope (Germany). The criteria for the identification of MN were according to Fenech [8].

Flow cytometric analysis of γ-H2AX foci. Peripheral blood mononuclear cells (PBC) were isolated by Histopaque-1077 density gradient centrifugation, washed twice with PBS and centrifuged at 900 x g for 10 min. The pellet containing PBC was suspended at a density of 5 x 10^6 cells/ml in RPMI 1640 medium supplemented with 20% foetal bovine serum, 10 µg/ml PHA, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, placed back into the incubator (37°C and 5% C02) and cultured for 0, 1 and 24 h. γ-H2AX foci were detected with the γ-H2AX (H2A.X PHOS) Detection Kit (Upstate Biotechnology, USA). Briefly, after incubation the cells were washed, fixed and resuspended in a permeabilization solution (0.5% saponin, 10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2). Unspecific binding was blocked for 1 hour in blocking buffer BSA-T-PBS (1 % BSA, 0.1% Triton X-100 in PBS). Thereafter, the cells were suspended in BSA–T–PBS containing 2 µg of the fluorescein-conjugated γ-H2AX antibody (monoclonal anti-phosphohistone H2AX antibody, Upstate Biotechnology) for 20 min. Cells were analyzed with a FACScan (Becton Dickinson, San Jose, CA, USA). 20,000 cells per point were analyzed for γ-H2AX intensity.

SNP genotyping. DNA was extracted from peripheral blood cells by the standard phenol-chloroform procedure. The amplifying and extension primers are shown in Table 2. The 4 SNPs were: *XRCC1* 194C>T (rs1799782), *XRCC1* 399G>A (rs25487), XRCC3 IVS5–14 A>G (rs rs1799796) and OGG1 326 Ser>Cys (rs1052134). Genotyping was performed according to the manufacturer's instructions. Briefly, multiple polymerase chain reaction (PCR) was performed according to the following programme: 94°C for 1 min, then 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min for 39 cycles. The PCR product was used for SNP extension: 96°C for 3 min, then 94°C for 20 sec and 40°C for 11 sec, for 45 cycles. A chip hybridization reaction was conducted at 42°C for 2 h with hybridization solution and hybridization additive solution at the proportion of 18:1. Chip data were scanned and analyzed using the SNPstream machine (Beckman Coulter, Inc.).

Flow cytometric analysis of apoptosis by the Annexin method. The frequencies of apoptotic and necrotic cells were detected with the Annexin V-FITC apoptosis detection Kit I (BD Pharmingen, USA), according to Darzynkiewicz [9]. Briefly, isolated PBC were washed twice with cold PBS, and then re-suspended in a 1X binding buffer at a concentration of 1x 10^6 cells/ml. 100 µl of cell suspension was incubated with 5 µl of Annexin V-FITC and 5 µl of PI (propidium iodide) at room temperature for 15 min in the dark. The cells were re-suspended in 400 µl of a 1 x binding buffer. The fluorescence was determined using a FACScan flow cytometer (Becton Dickinson). A computer system (CellQuest Pro, Becton Dickinson) was used for data acquisition and analysis. Data for 20,000 events were stored. A cell gate containing lymphocytes was established on the basis of forward and side-light scatter.

Statistical analysis. Performed using Statistica 7.1 software (Stat Soft. Inc., Tulsa, OK, USA). The one-way analysis of variance (ANOVA) or unpaired t-test was used to compare the results between cancer patients and healthy donors. Pearson correlation coefficients were used to look for associations between MN frequencies, intensity of fluorescence signal and apoptosis and age. The significance of the differences of observed alleles and genotypes between groups was tested using χ^2 analysis. Significant differences were defined at

Table 2. Technical details of PCR-RFLP analysis

GenE	xrcc1	XRCC1	XRCC3	OGG1
POLYMORPHISM	Arg194Trp	Arg399Gln	IVS5-14 17.893	Ser326Cys
PRIMER F	GTTCCGTGTGAAGGAGGAG	CTGGACTGCTGGGTCTGAG	GACACCTCTACAGAGGACG	GTGGATTCTCATTGCCTTCG
PRIMER R	CTTGGAGGTGCTGCCTATG	CTCCAGATTCCTGGCATTGC	TTCTCGATGGTTAGGCACAG	CTGTTGCTGTCGAGACTGC
RESTRICTION ENZYME	PvuII	HpaII	PvuII	Fnu4HI
AMPLIFICATION CONDITIONS	1.95°C – 2 min 2. 95°C – 20 s × 12 cykli 3. 67°C – 15 s 4. 72°C – 1 minuta 5. 95°C – 40 s × 24 cykle 6. 55°C – 40 s 7. 72°C – 30 s 8. 72°C – 10 minut	1. 95°C – 2 min 2. 95°C – 20 s × 12 cykli 3. 69°C – 15 s 4. 72°C – 1 minuta 5. 95°C – 40 s × 24 cykle 6. 55°C – 40 s 7. 72°C – 30 s 8. 72°C – 10 minut	1. 95°C – 5 min 2. 95°C – 1 minuta × 35 cykli 3. 58°C – 1 minuta 4. 72°C – 1 minuta 5. 72°C –10 minut	1. 95°C – 5 min 2. 95°C – 1 minuta × 35 cykli 3. 58 °C – 1 minuta 4. 72 °C – 1 minuta 5. 72 °C – 10 minut
PCR PRODUCT SIZE (bp)	504	849	650	672
FRAGMENTS IDENTIFYING GENOTYPES (bp)	CC=431 CT=368+431 TT=368	GG=321+461 GA=321+461+528 AA=321+528	AA=283+367 AG=283+367+650 GG=650	CC=553 CG=154+399+553 GG=154+399

$p< 0.05$. The odds ratio (ORs) and 95% confidence intervals (CIs) were calculated using a logistic regression model. The *post-hoc* power of the study was performed according to Faul [10] and Rosner [11].

RESULTS

Micronucleus assay. The mean spontaneous MN yield (10.1±1.14) and radiation–induced MN yield (91.5±11.4) in prostate cancer patients was significantly higher (p=0.046; p=0.025, respectively) as compared to the mean spontaneous MN yield (7.3±1.6) and radiation-induced MN yield (64.4±11.9) in male healthy donors (Figs. 1A, 1B). The mean spontaneous MN yield in cervical cancer patients (16.1±2.34) was not significantly different (p=0.162; Cohen's d=– 0.41, power=0.34) from the yield in healthy donors (11.3±2.1) (Fig. 1C). The mean radiation-induced MN yield in cervical cancer patients (117.5±11.72) was significantly higher (p=0.008), compared to that of healthy donors (69.2±10.2) (Fig. 1D). There was no effect of donor's age on the spontaneous and radiation-induced MN frequencies in cells of cervical cancer patients (p=0.162; p=0.602, respectively), prostate cancer patients (p=0.465; p=0.072, respectively), female healthy donors (p=0.152; p=0.693, respectively) and male healthy donors (p=0.245; p=0.720, respectively). The frequency of micronuclei in prostate and cervical cancer was independent of cancer stage, based on the TNM/Gleason classification and the TNM/FIGO classification.

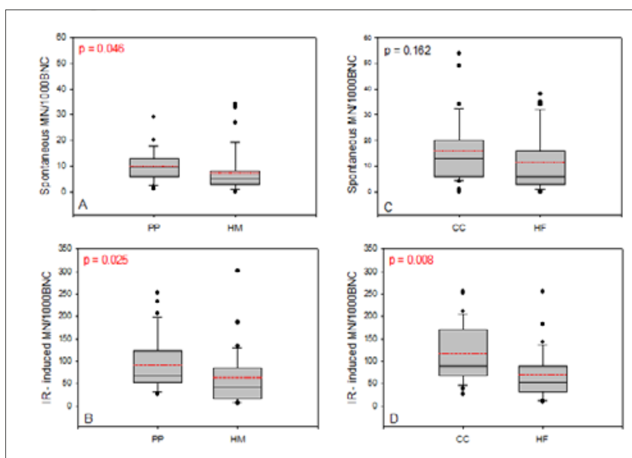

Figure 1. Spontaneous and radiation-induced frequency of MN in prostate cancer patients (A, B), cervical cancer patients (C, D) and healthy donors, Data are presented as box plots with a horizontal line at the median and a dotted line at the mean value. Statistical significance – p < 0.05.
HM – healthy male donors;
PP – prostate cancer patients;
HF – healthy female donors,
CC – cervical cancer patients.

γ-H2AX assay. The expression of γ-H2AX foci, measured as a mean intensity of fluorescence, was detected before irradiation as well as 1 and 24 h after irradiation. The mean intensities of spontaneous and radiation-induced γ-H2AX foci fluorescence in prostate cancer patients 1h and 24 h after exposure were not significantly different from the corresponding groups of healthy donors:
30.73±3.57 vs. 30.05±2.35; p=0.615;
96.41±8.32 vs. 94.95±7.88; p=0.899;
61.98±7.1 vs. 53.52±3.76; p=0.975 (Figs. 2 A, 2 B, 2 C).

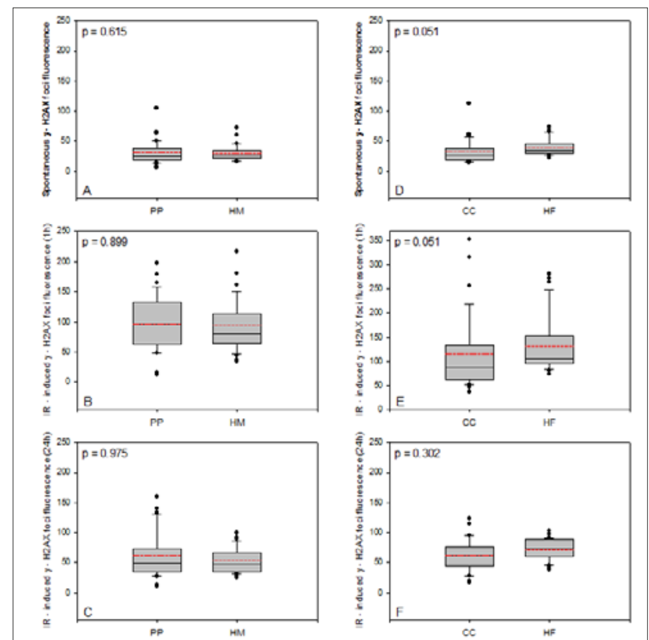

Figure 2. Intensity of spontaneous and radiation-induced γ-H2AX foci fluorescence 1 and 24 hours after irradiation in prostate cancer patients (A, B, C), cervical cancer patients (D, E, F) and healthy donors. Data are presented as box plots with a horizontal line at the median and a dotted line at the mean value. Statistical significance – p < 0.05.
HM – healthy male donors;
PP – prostate cancer patients;
HF – healthy female donors,;C
C – cervical cancer patients.

The mean intensities of spontaneous and radiation-induced γ-H2AX foci fluorescence in cervical cancer patients 1h and 24 h after exposure were not significantly different from the corresponding groups of healthy donors:
32.18±3.75 vs 39.90±2.63; p=0.051;
116.85±14.04 vs. 130.75±10.80; p=0.051;
62.44±4.83 vs. 70.97±3.17; p=0.302, respectively. (Figs. 2 D, 2 E, Fig. 2 F).

The capacities of disappearance of γ-H2AX foci fluorescence (24 h vs. 1 h) in cervical and prostate cancer patients (51.97 %±4.96 and 49.14 %±4.67, respectively) were not significantly different, compared to the corresponding groups of healthy donors (56.78 %±3.82, p=0.571and 57.9% ±3.52, p=0.893, respectively) (Figs. 3 A, 3 B). There was no effect of donor's age on the intensity of spontaneous γ-H2AX foci fluorescence in cells of cervical cancer patients (p=0.288), prostate cancer patients (p=0.109), female healthy donors (p=0.513) and male

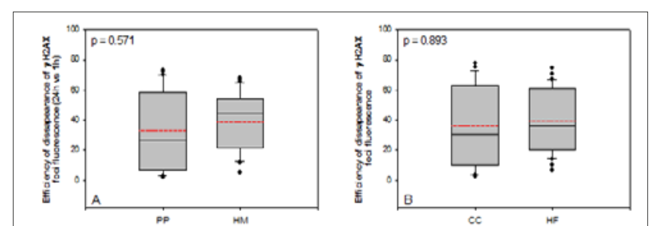

Figure 3. Capacity of disappearance of γ-H2AX foci fluorescence in prostate cancer patients (A), cervical cancer patients (B) and healthy donors (24 h vs. 1 h). Data are presented as box plots with a horizontal line at the median and a dotted line at the mean value. Statistical significance – p < 0.05.
HM – healthy male donors;
PP – prostate cancer patients;
HF – healthy female donors;
CC – cervical cancer patients).

Table 3. Genotype and allele distribution in prostate cancer patients and healthy male donors

Codon	Genotypes and alleles	Prostate cancer patients		Healthy donors		OR (95% CI)	p	power
		n	%	n	%			
XRCC1 194C>T	CC	27	90.0	24	80.0	2.250 (0.507 – 9.994)	0.235	0.1131
	CT	3	10.0	6	20.0	0.444 (0.100 – 1.974)	0.235	0.1131
	TT	0	-	0	-	-	-	-
	C	57	95	54	90.0	2.111 (0.502 – 8.866)	0.245	0.0315
	T	3	5	6	10.0	0.473 (0.112 – 1.989)	0.245	0.0315
XRCC1 399G>A	GG	18	60.0	10	33.3	3.000 (1.046 – 8.602)	0.034*	0.4632
	GA	10	33.3	18	53.3	0.333 (0.116 – 0.955)	0.034*	0.4117
	AA	2	6.7	2	6.7	1.000 (0.131 – 7.604)	0.694	0.0414
	G	46	76.7	38	63.3	1.902 (0.858 – 4.216)	0.081	0.1537
	A	14	23.3	22	36.7	0.525 (0.237 – 1.165)	0.081	0.1543
XRCC4 IVS5-14 A>G	AA	7	23.3	7	23.3	1.000 (0.302 – 3.309)	0.619	0.0489
	AG	19	63.3	20	66.7	0.864 (0.299 – 2.498)	0.500	0.0567
	GG	4	13.3	3	10.0	0.385 (0.282 – 6.796)	0.500	0.1802
	A	33	55	34	56.7	0.934 (0.454 – 1.921)	0.499	0.0533
	G	27	45	26	43.3	1.069 (0.520 – 2.199)	0.499	0.0532
OGG1 326 Ser>Cys	CC	20	66.7	17	56.7	0.352 (0.083 – 1.494)	0.130	0.5162
	CG	10	33.3	10	33.3	1.000 (0.342 – 2.926)	0.607	0.0479
	GG	0	-	3	10.0	-	-	-
	C	50	83.3	44	73.3	1.818 (0.748 – 4.418)	0.133	0.1245
	G	10	16.7	16	26.7	0.550 (0.226 – 1.336)	0.133	0.1245

healthy donors (p=0.192) There was also no effect of donor's age on the intensity of radiation induced γ-H2AX foci fluorescence 1 and 24 h after irradiation in cells of cervical cancer patients (p=0.540 and p=0.180, respectively), prostate cancer patients (p=0.160 and p=0.235, respectively), female healthy donors (p=0.936 and p=0.501, respectively), and male healthy donors (p=0.268 and p=0.890, respectively). The intensities of γ-H2AX foci fluorescence in prostate and cervical cancer were independent of cancer stage, based on the TNM/Gleason classification and the TNM/FIGO classification.

***XRCC1* 194C>T, *XRCC1* 399G>A, XRCC3 IVS5–14 A>G (rs rs1799796) and OGG1 326 Ser>Cys polymorphisms.** Except for the XRCC3 (IVS5–14) genotype in cervical cancer patients, the genotype distributions in all groups were consistent with the Hardy-Weinberg equilibrium distribution. There was no statistical difference in the genotypes of the *XRCC1* 194C>T, XRCC3 IVS5–14 A>G and OGG1 326 Ser>Cys between prostate cancer patients and healthy donors (Tab. 3). However, increased prostate cancer risk was observed for men with the XRCC1 *399G>A* GG genotypes [OR 3.00 (95% CI 1.046–8.602); p=0.034]. A significant negative association was found between the XRCC1 *399G>A* AG genotype and prostate cancer risk [OR 0.333 (95% CI 0.116–0.955), p=0.034]. There was no statistical difference in the genotypes of the *XRCC1* 194C>T, *XRCC1* 399G>A, XRCC3 IVS5–14 A>G and OGG1 326 Ser>Cys between cervical cancer patients and healthy donors (Tab. 4).

Apoptosis. The level of spontaneous and radiation-induced apoptosis 24 h post-exposure was determined (Fig. 4). The levels of spontaneous and radiation-induced apoptosis in prostate cancer patients were not significantly different compared with healthy donors (11.79±6.23 vs. 15.36±10.91; p=0.367and 34.54±5.93 vs. 33.43±10.40; p=0.994, respectively) (Figs. 4 A, 4 B). The level of spontaneous apoptosis, but not radiation-induced apoptosis in cervical cancer patients was significantly lower, compared to healthy donors (8.75±1.78 vs. 13.89±7.04; p=0.004 and 25.47±2.27 vs. 30.42 ±8.07; p=0.145, respectively) (Figs. 4 C, 4 D). There was no effect of donor's age on the spontaneous and radiation-induced apoptosis in cells of cervical cancer patients (p=0.158 and p=0.162, respectively), prostate cancer patients (p=0.784 and p=0.520, respectively), female healthy donors (p=0.417 and p=0.169, respectively), and male healthy donors (p=0.952 and p=0.104, respectively). The frequencies of apoptotic cells in prostate and

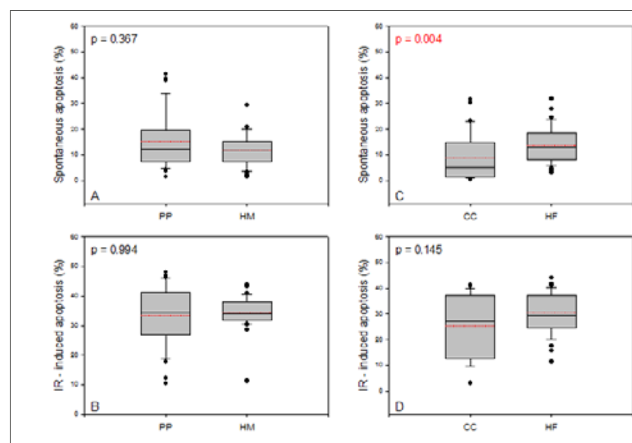

Figure 4. Frequency of spontaneous and radiation-induced apoptosis in prostate cancer patients (A, B), cervical cancer patients (C, D) and healthy donors. Data presented as box plots with a horizontal line at the median and a dotted line at the mean value. Statistical significance – $p < 0.05$.
HM – healthy male donors;
PP – prostate cancer patients;
HF – healthy female donors;
CC – cervical cancer patients.

Table 4. Genotype and allele distribution in cervical cancer patients and healthy female donors

Codon	Genotypes and alleles	Cervical cancer patients		Healthy donors		OR (95% CI)	p	power
		n	%	n	%			
XRCC1 194C>T	CC	27	90.0	23	76.6	2.739 (0.635 – 11.823)	0.149	0.1370
	CT	3	10.0	7	12.3	0.365 (0.085 – 1.576)	0.149	0.1370
	TT	0	-	0	-	-	-	-
	C	57	95	53	88.3	2.509 (0.616 – 10.209)	0.161	0.0344
	T	3	5	7	11.7	0.398 (0.097 – 1.621)	0.161	0.0344
XRCC1 399G>A	GG	16	53.3	14	46.7	1.306 (0.474 – 3.602)	0.398	0.0807
	GA	11	36.7	12	40.0	0.868 (0.306 – 2.461)	0.500	0.0552
	AA	3	10.0	4	13.3	0.722 (0.147 – 3.554)	0.500	0.0654
	G	43	71.7	40	66.7	1.264 (0.581 – 2.750)	0.346	0.0670
	A	17	28.3	20	33.3	0.719 (0.363 – 1.719)	0.346	0.0836
XRCC4 IVS5-14 A>G	AA	7	23.3	9	30.0	0.710 (0.225 – 2.246)	0.385	0.0804
	AG	21	70.0	16	53.5	2.042 (0.707 – 5.895)	0.144	0.2065
	GG	2	6.7	5	16.7	0.357 (0.064 – 2.007)	0.211	0.0697
	A	35	58.3	34	56.7	1.070 (0.519 – 2.208)	0.499	0.0511
	G	25	41.7	26	43.3	0.934 (0.452 – 1.926)	0.499	0.0511
OGG1 326 Ser>Cys	CC	18	60.0	23	76.7	0.457 (0.149 – 1.396)	0.133	0.3303
	CG	10	33.3	6	20.0	2.000 (0.619 – 6.465)	0.190	0.2575
	GG	2	6.7	1	3.3	2.071 (0.178 – 24.150)	0.500	0.1318
	C	46	76.7	52	86.7	0.505 (0.194 – 1.313)	0.118	0.2196
	G	14	23.3	8	13.3	1.978 (0.761 – 5.140)	0.118	0.2191

cervical cancer were independent of cancer stage, based on the TNM/Gleason classification and TNM/FIGO classification.

DISCUSSION

The presented study investigated the role of chromosomal damage, capacity of DSB repair, polymorphisms in DNA repair genes and apoptosis as predictive markers of the risk of prostate and cervical cancer. The extent of chromosomal damage was evaluated by the cytokinesis-block micronucleus assay, which is a comprehensive system for measuring DNA damage, cytostasis and cytotoxicity [12]. The results obtained revealed an enhanced frequency of spontaneous and radiation-induced micronuclei in prostate and cervical cancer patients, compared to healthy donors. However, the association between increased frequency of micronuclei and risk of developing prostate and cervical cancer was independent of the cancer stage, based on the the TNM/Gleason classification and TNM/FIGO classification. This observation is consisted with results published by other authors who also observed a higher frequency of micronuclei in lymphocytes of prostate cancer patients, compared to healthy donors [13]. Similar to these data, Chakrabarti [14] and Cerequeira [15] reported elevated frequencies of spontaneous micronuclei in epithelial cells of cervical cancer patients compared to healthy donors. Interestingly, Leal-Garza [16] even observed a direct association between the frequency of MN in epithelial cells of cervical cancer patients with the stage of progression of cervical cancer, a finding not confirmed by others. Furthermore, in the current study, no significant correlation was found between donor age and frequency of micronuclei in ymphocytes of prostate and cervical cancer patients and healthy donors. This result is consistent with the data published by several authors [17]. However, there are also results indicating such correlation [18] but the reason for the controversy is not clear. Current evidence suggests that chromosomal instability and cancer susceptibility can be promoted by failure or 'mis-repair' of DNA double-strand breaks (DSBs), which are repaired by non-homologous end-joining (NHEJ) and homologous recombination (HR) [19]. The presented study determined the initial level of DSBs and DSB repair kinetics, measured by the γ-H2AX assay, which is a sensitive biomarker to assess such lesions [20]. The intensity of γ-H2AX foci fluorescence was detected before *in vitro* irradiation, as well as 1 and 24 h after irradiation. Flow cytometry was used in this study because it is a more acceptable method in clinical studies than the measurement of the number of γ-H2AX foci by fluorescence microscopy [21]. The presented data showa that the intensities of spontaneous and radiation-induced γ-H2AX foci fluorescence 1 h after exposure in cells from prostate and cervical cancer patients were not significantly different from those in the cells of healthy donors. After 24 h, the intensities of γ-H2AX foci fluorescence were reduced in all 4 groups of donors, indicating progression of DSB repair. It is interesting to note that the activation of the DNA damage response and the processing of DNA damage in prostate and cervical cancer patients was not significantly different from the corresponding groups of healthy donors. Moreover, the distribution of the patient's age and cancer stage did not influence the γ-H2AX levels.

To-date, few papers have investigated the usefulness of γ-H2AX foci fluorescence as a biomarker of cancer susceptibility. Recently, Brzozowska [22] observed no significant differences between the intensity of the spontaneous γ-H2AX foci fluorescence in lymphocytes of prostate cancer patients and healthy men. However, a 1.7-fold higher initial fluorescence signal was observed 0.5 h after exposure in healthy

donors compared to prostate patients. Kotsopoulos [23] found no significant differences in the spontaneous number of γ-H2AX nuclear foci between breast cancer patients and healthy donors and following γ-irradiation. In contrast, Xu [24] reported that radiation-induced a γ-H2AX level and the ratio of induced γ-H2AX level to baseline level was associated with an increased risk of esophageal adenocarcinoma. However, Fernandez [25] showed that the basal and radiation-induced γ-H2AX levels in peripheral blood lymphocytes is a suitable risk predictor for bladder cancer.

The current state of the literature supports a prominent role for apoptosis in cancer development, and suggests an association between genetic variants in the apoptotic pathway and cancer risk [26]. It was therefore interesting to test the hypothesis that an attenuation of apoptosis could contribute to the development of prostate and cervical cancers. The authors of the current study determined the level of spontaneous as well as radiation-induced apoptosis 24 h post-exposure with the Annexin V-FITC assay, and found no significant differences between prostate cancer patients and healthy donors. However, the level of spontaneous apoptosis in cervical cancer patients was significantly lower, compared to healthy donors. To- date, there are relatively few studies which investigate the association between the level of apoptosis and cancer risk. Crompton [27] found no significant difference between the level of apoptosis in the lymphocytes of healthy donors and patients with various types of cancer. Similar results were observed by Docherty [28], who found no significant differences in apoptotic response to ionizing radiation between breast cancer patients and healthy donors. Brzozowska [22], however, reported no significant differences in apoptotic response between prostate cancer patients and healthy donors. In contrast, unselected breast cancer patients have previously been shown to have a significantly lower apoptotic response to 4 Gy irradiation than controls [29].

DNA repair is a complex process involving a number of DNA repair pathways that rely on the products of many genes. Previous studies revealed an association between single-nucleotide polymorphisms (SNPs) in DNA repair genes and the risk of cancer [30]. However, the results of these studies are not consistent due to different patient populations, cancer types and case selections. Therefore, the current study aimed to investigate the association between genetic variants of XRCC1 (194 Arg/Trp), XRCC1 (399 Arg/Gln), XRCC3 (IVS5–14), OGG1 (326 Ser/Cys) and risk of prostate and cervical cancer. These DNA repair gene polymorphisms, representing different repair pathways have been shown to be involved in cancer predisposition. The results obtained revealed a strong association between the polymorphism of the the XRCC1 (399 Arg/Gln) gene and risk of prostate cancer. The variant genotype Arg/Arg was significantly associated with a higher risk of prostate cancer when compared to the wild-type genotype with an adjusted OR (95%CI) of 3,000 (1.046 – 8.602; p=0.034), whereas the heterozygous variant Arg/Gln) was markedly associated with a decreased risk of prostate cancer [OR=0.333 (0.116–0.955); p=0.034]. These results are consistent with the result of van Gils [31] who reported that the risk of prostate cancer decreased significantly in heterozygous variants (Arg/Gln) in the XRCC1 (399 Arg/Gln) gene. Rybicki [32] showed that prostate cancer risk increased in homozygous variants (Arg/Arg) in conjunction with the XPD gene polymorphism (312 Asn/Asn). Similar results were published by Ritchey [33] who observed a higher risk of prostate cancer in homozygous variants (Arg/Arg). However, Chen [34] reported that the variant Gln/Gln homozygote might be a risk factor for prostate cancer. In contrast, Hirata [35] showed no significant association between gene polymorphism of XRCC1 (399 Arg/Gln) and the risk of prostate cancer. Interestingly, when the results of all these 7 reports were pooled in a meta-analysis, no significant associations were observed between *XRCC1* 399 Arg/Gln polymorphism and the risk of prostate cancer in worldwide populations [36]. However, in the stratified analysis by ethnicity, these results indicated a significant association of *XRCC1* Arg399Gln polymorphism with prostate cancer risk in Asian subjects. In the presented study, no significant association was found between the XRCC1 (194 Arg/Trp), OGG1 (326 Ser/Cys) and XRCC3 (IVS5–14) polymorphisms and an increased risk of prostate cancer. To-date, epidemiological studies in different populations have shown inconsistent association between these polymorphisms and susceptibility to prostate cancer. The role of OGG1 (326 Ser/Cys) polymorphism in susceptibility to prostate cancer was assessed by other authors [5], who revealed that the OGG1(326 Ser/Cys) variant allele distribution was not significantly different from those in the controls and did not demonstrate association with prostate cancer. In contrast, it was demonstrated that the OGG1 326 Ser/Cys polymorphism increased the susceptibility to prostate cancer [37]. For the XRCC3 (IVS5–14) polymorphism there is only one study which shows no association with prostate cancer risk in an Indian population [38]. The presented study also indicates that the XRCC1 (399 Arg/Gln), XRCC1 (194 Arg/Trp), XRCC3 (IVS5–14) and OGG1 (326 Ser/Cys) polymorphisms are not associated with cervical cancer. The observations in the current study are comparable to a meta-analysis by Mei [39], who did not observe any significant association between the XRCC1 (399 Arg/Gln) and the XRCC1 (194 Arg/Trp) polymorphisms, and overall cervical cancer risk. For the OGG1 (326 Ser/Cys) polymorphism, there are only 2 studies, bot of which show no association with cervical cancer risk [40, 41]. To the best of the knowledge of the authors of the presented srtudy, currently there are no data dealing with association between the XRCC3 (IVS5–14) polymorphism and cervical cancer risk.

CONCLUSIONS

The results obtained demonstrate that distinct factors may be uniquely predictive for prostate and cervical cancers. Among all tested factors, only the homozygous variant (Arg/Arg) in XRCC1 (399 Arg/Gln) was strongly associated with prostate cancer risk, and only a significantly lower apoptotic response was connected with cervical cancer risk. The presented study supports a positive association between the frequency of MN and increased prostate cervical cancer risk. However, there is a significant limitation of such a biomarker as it is not cancer specific. The results also indicate that the information obtained by analyzing the spontaneous and radiation-induced γ-H2AX foci fluorescence and apoptosis is of no value for predicting prostate and cervical cancers. The power of this study for the γ-H2AX assay, however, was too small to enable any definitive statements to be made. The final conclusion of this study is that cancer susceptibility is a complex phenotype not readily detectable in relatively small studies by functional assays or analysis of SNP in a few, selected genes.

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