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Association of DNA repair gene polymorphisms with genotoxic stress in underground coal miners

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Abstract

In underground coal mining, numerous harmful substances and ionising radiation pose a major threat to the occupational safety and health of workers. Because cell DNA repair machinery eliminates genotoxic stress conferred by these agents, we examined whether single nucleotide polymorphisms in h*OGG1* (rs1052133), *XRCC1* (rs25487), *ADPRT* (rs1136410), *XRCC4* (rs6869366) and *LIG4* (rs1805388) genes modulate the genotoxic damage assessed by the cytokinesis-block micronucleus assay in lymphocytes from 143 underground coal miners and 127 healthy non-exposed males. We also analyzed models of gene–gene interactions associated with increased cytogenetic damage in coal miners and determined 'protective' and 'risk' combinations of alleles. We showed that miners with the G/G genotype of the h*OGG1* (rs1052133) gene had a significantly increased frequency of binucleated lymphocytes with micronuclei (13.17‰, 95% CI = 10.78–15.56) compared to the C/C genotype carriers (10.35‰, 95% CI = 9.59–11.18). In addition, in the exposed group this indicator was significantly increased in carriers of the T/T genotype of the *LIG4* (rs1805388) gene compared to miners harbouring the C/T genotype (13.00‰, 95% CI = 10.96–15.04 and 9.69‰, 95% CI = 8.32–11.06, respectively). Using the multifactor dimensionality reduction method, we found the three-locus model of gene–gene interactions h*OGG1* (rs1052133) × *ADPRT* (rs1136410) × *XRCC4* (rs6869366) associated with high genotoxic risk in coal miners. These results indicate that the studied polymorphisms and their combinations are associated with cytogenetic status in miners and may be used as molecular predictors of occupational risks in underground coal mines.

Introduction

Coal is the largest fossil fuel source used for the generation of energy (1), and its extraction is prominent all over the world. However, workers of industrial enterprises are exposed to a wide range of occupational hazards that can lead to genome instability and various diseases. Indeed, during underground coal mining, large concentrations of coal dust particles and polycyclic aromatic hydrocarbons (PAHs) are released. Underground coal mines are also characterised by high levels of ionising radiation (2–4). Further, coal waste is a major source of ash, soot and heavy metals (5). Collectively, exposure to these agents is hazardous due to their synergistic, additive and enhancing effects (6). Inhalation of complex mixtures containing substances such as heavy metals, ash, iron, PAHs and sulphur has

been reported to result in genotoxic damage (1,5,7) and development of pulmonary diseases like coal workers' pneumoconiosis, progressive massive fibrosis, bronchitis, lung failure, emphysema and lung cancer (8,9).

Individual susceptibility to genotoxic environmental and occupational factors is determined by single nucleotide polymorphisms (SNPs). SNPs result in amino acid substitutions that can influence protein function (10), lead to altered splicing (11) or affect coding regions, including the disruption of exonic splicing enhancer sequences(12) or exonic mRNA stability/instability sequences (13). In promoter regions, SNPs can alter transcription factor binding motifs, change the efficiency of enhancer or repressor elements (14) or introduce an alternative translation initiation codon, leading to downregulation of the transcript (15). Thus, SNPs in genes involved in response to genotoxic stress can affect their activity and efficiency.

DNA repair is a crucial function of cells and the main mechanism which eliminates DNA damage occurring upon the exposure to genotoxic factors. Every spontaneous alteration of DNA sequence might lead to the expression of defective cell components that are unable to properly function. Malfunction of the DNA repair machinery leads to cell death via necrosis and apoptosis. Mammalian cells develop 10^4–10^5 DNA single-strand breaks per day, which all must be quickly and accurately repaired (16). Unrepaired DNA damage can lead to cytogenetic abnormalities, such as micronuclei (MN) (17). MN are found in dividing cells that either contain chromosome breaks (acentric fragments), and/or whole chromosomes that are unable to travel to the spindle poles during mitosis (18). The frequency of micronucleated cells provides a convenient and reliable index of chromosome breakage and loss.

The cytokinesis-block micronucleus (CBMN) assay of blood lymphocytes is a well-recognised cytogenetic technique used for the assessment of DNA damage induced by exposure to genotoxic factors (19). Besides MN, this method enables to record nucleoplasmic bridges (NPBs) as a marker of dicentric chromosomes; protrusions (NBUDs), representing a mechanism by which cells remove amplified DNA; and nuclear division index (NDI), which reflects the proliferation activity of cells (19–22).

In this study, we examined whether SNPs in DNA repair genes h*OGG1* (rs1052133), *XRCC1* (rs25487), *ADPRT* (rs1136410), *XRCC4* (rs6869366) and *LIG4* (rs1805388) are associated with genotoxic damage, assessed by the CBMN assay in underground coal miners. We also analyzed the model of gene–gene interactions associated with the increased frequency of cytogenetic damage in coal miners to determine the 'protective' and 'risk' allele combinations.

Material and methods

Group characteristics

Blood samples were collected from 143 coal miners (Caucasian males) working in underground coal mines (Kemerovo Region, Russian Federation) and undergoing medical examination at the Research Institute for Complex Problems of Hygiene and Occupational Diseases (Novokuznetsk, Kemerovo Region, Russian Federation). The mean length of service in coal mines was 23.26 ± 9.66 years; mean age of donors included in the exposed group was 50.11 ± 7.36 years. As a control group, we used blood samples collected from 127 healthy unexposed men (donors of the Kemerovo Centre for Blood Transfusion, Kemerovo, Russian Federation). The mean age in the control group was 47.67 ± 8.45 years. A full description of donors included in this study is presented in Table 1.

Coal miners were matched to non-exposed males by ethnicity, age, social-economic status and dietary habits. All donors were interviewed about their health conditions and smoking status, cancer history, drug intake, allergies, length of service, previous X-rays examinations or other medical treatments.

Exclusion criteria for the study were age over 60 years, intake of drugs with known mutagenic effects, receiving an X-ray examination up to 3 months prior to participation in this study, infectious and inflammatory diseases or cancer. All participants were informed about the aim, methodology and possible risks of the study; informed consent was signed by each donor. The design of this study was approved by the local ethics committee of the Kemerovo State University.

Cytogenetic investigation

The degree of DNA damage was accessed by the routine protocol of CBMN (23,24) with modifications given by Ingel (25). The whole blood was collected in vacutainers with heparin by vein puncture of the ulnar vein and then was stored at 4°C up to 24 h. 0.2 ml blood, 3 ml RPMI-1640 (PanEco Ltd., Moscow, Russian Federation), 0.8 ml foetal bovine serum (PanEco Ltd, Moscow, Russian Federation) and 30 µl phytohaemagglutinin (PanEco Ltd, Moscow, Russian Federation) were incubated in culture flasks at 37°C. After for 44 h cultivation, cytochalasin B (Applichem GmbH, Germany) was added into each culture flask at a final concentration of 6 µg/ml and incubated for another 24 h at 37°C. Then, cultures were centrifuged for 10 min at 1000 rpm, the supernatant was detached, and the pellet was resuspended in 6 ml of an ice-cold, freshly prepared 0.125 M KCl (Helicon, Moscow, Russian Federation) solution. Pre-fixation was performed using 1 ml of ice-cold, freshly prepared Carnoy's fixative (methanol and acetic acid in a ratio of 3:1). The pellet was resuspended, and the suspension was centrifuged for 10 min at 1000 rpm. The supernatant was detached, the pellet was resuspended in another 10 ml of ice-cold Carnoy's fixative and left for 1 h at +4°C. This step was repeated several times until the pellet appeared clean and the cell suspension was clear. The sample was pipetted onto dry, ice-cold glass slides. The slides were encoded, stained with 2% Giemsa solution (PanEco Ltd., Moscow, Russian Federation) for 15 min and analyzed using a Nikon Eclipse 80i microscope with transmitted light and a full filter at 1000× magnification.

On each slide, 1000 binucleated (BN) lymphocytes per individual were analyzed, and MN, NPBs and NBUDs were scored according

Table 1. Age, length of service and smoking status in the studied groups

Group	Number	Age, years		Length of service in coal mining conditions, years		Smoking status		
		µ ± SD	Min–max	µ ± SD	Min–max	Smokers	Non-smokers	Ex-smokers
Exposed	143	50.11 ± 7.36	24–60	23.26 ± 9.66	4–39	56	64	23
Non-exposed control	127	47.67 ± 8.45	25–60	0	0	49	50	28

to criteria described by Fenech (19,23). The NDI was calculated using the accepted recommendations (26).

DNA extraction

DNA was extracted using the routine phenol/chloroform method. 2 ml of whole blood was transferred into 15 ml Falcon centrifuge tubes containing 10 ml of ice-cold sucrose buffer [320 mM sucrose (Helicon, Moscow, Russian Federation), 5 mM $MgCl_2$ (Ameresco, USA), 10 mM Tris–HCl (Ameresco, USA) and 1% Triton X-100 (Ameresco, USA)]. The samples were mixed and left for 1 h at +4°C. Then, tubes were centrifuged for 20 min at 4000 rpm with cooling to 0°C, the supernatant was detached, and 0.3 ml of SE buffer (SibEnzyme Ltd., Novosibirsk, Russian Federation) was poured to the each tube. The pellet was resuspended and transferred into Eppendorf tubes. Ten percent SDS buffer (30 µl) and 7.5 µl of proteinase K (Thermo Fisher Scientific Inc., USA) were added to each Eppendorf tube. The samples were vigorously shaken and incubated at +37°C. After 24 h of incubation, 350 µl of phenol was added to each tube; samples were vigorously shaken and centrifuged for 6 min at 9000 rpm. The upper, aqueous phase was transferred to another Eppendorf tube, and one volume of phenol/chloroform (1:1) solution (approximately 300 µl) was added into each tube, the samples were vigorously shaken and centrifuged for 6 min at 9000 rpm. Upper, aqueous phase was transferred to another Eppendorf tube, and the previous step was repeated. Then one volume of chloroform (approximately 300 µl) was added to the aqueous phase, tubes were vigorously shaken and centrifuged for 6 min at 9000 rpm. Finally, DNA was precipitated using a 17 µl of 4M NaCl (Helicon, Moscow, Russian Federation) and 700 µl of cold 90% ethanol (27).

Polymerase chain reaction

In our research, we used the following criteria for SNP selection: location within genes associated with mechanisms of MN formation, minor allele frequency is ≥5% for Caucasians population, functional consequence and no studies on the role of the SNPs in individual susceptibility of coal miners. Detection of SNPs in the genes encoding base excision repair (BER) and double-strand breaks repair (DSBR) proteins (Table 2) was performed using reagent kits produced by Lytech Ltd. (Moscow, Russian Federation) by allele-specific polymerase chain reaction (PCR). The 0.5 ml amplification tubes were numbered; the reagents for PCR were thawed for 20–30 min before use. We prepared the amplification solution immediately before the experiment. 17.5 µl of diluent, 2.5 µl of the reaction mixture and 0.2 µl of Taq-polymerase were used for each sample. We prepared two working mixtures: reaction N (normal) and reaction P (pathology) corresponding to wild-type and mutant alleles, respectively. After preparation of working mixtures, 25 µl of mineral oil was poured into each tube. Next, 5 µl of DNA samples were taken for analysis and 5 µl of the diluent were added in negative control tube. The samples were centrifuged for 3–5 s at 1500–3000 rpm at 25°C. The tubes were then placed into a thermocycler and the amplification was performed according to a program suggested by manufacturer (Table 3).

Detection of results was performed using separation of amplification products by horizontal electrophoresis in a 3% agarose gel. 100 ml of melted and cooled to +50–+60°C agarose was poured into the tray used for loading of gel in electrophoresis chamber. Next, 8–10 µl of the amplified PCR product was placed into gel pockets. The electrophoresis was conducted in conditions of intensity of the electric field at 10–15 V per cm of gel. Staining was performed using 10 µl of 1% ethidium bromide solution. Results of electrophoresis were detected using UV-transilluminator Vilber Lourmat ECX-F15.C (Vilber Lourmat GmbH, Germany). Image capture was performed by a photo camera and the computer software GelImager (PanEco Ltd., Moscow, Russian Federation).

Statistical analysis

Statistical analysis was performed using StatSoft STATISTICA 7.0 and SPSS Statistics 17.0 software. We used the Kolmogorov–Smirnov test to verify the compliance of the data with the normal distribution. For quantitative data, the mean and 95% confidence interval (95% CI) were calculated. Significant differences between groups were defined with the Mann–Whitney *U*-test. To avoid the effect of multiple comparisons, false discovery rate (FDR) correction was applied. The differences were statistically significant if $P < 0.05$. We used receiver operating characteristic (ROC) analysis (calculation of the AUC index) to assess the predictive significance of models. Conformity to Hardy–Weinberg equilibrium was determined by χ^2 analysis. To identify the associations of gene polymorphism and cytogenetic damage with regard to quantitative (age, length of service) and binary (smoking status, ethnicity) factors, Poisson regression was calculated. Coefficient of regression was interpreted as the odds ratio (OR) for Poisson model taking into account all the variables included in the regression equation. For OR, 95% CI was calculated. The models of gene–gene interactions and the 'protective' and 'risk' alleles' combinations were determined by multifactor dimensionality reduction (MDR) method using MDR 3.2.0 software.

Results

Previously, we demonstrated a significant increase in the frequency of BN lymphocytes with MN, NPBs and NBUDs and the reduction

Table 2. Characteristic of the studied polymorphisms

Gene	Reference SNP ID number (loci)	Primers (5'→ 3')
Base excision repair		
h*OGG1*	rs1052133	F: 5'-ggaaggtgcttggggaat-3'
	(c.977C>G, p.Ser326Cys)	R: 5'-actgtcactagtctcaccag-3'
XRCC1	rs25487	F: 5'-gaatgccctgatcgctatctca-3'
	(c.1196A>G, p.Arg399Gln)	R: 5'-gttgccctcatttcacggcgag-3'
ADPRT	rs1136410	F: 5'-tgctgcctatacagtcacttt-3'
	(c.2285T>C, p.Val762Ala)	R: 5'-gtggccatcacattcgtcagat-3'
Double-strand breaks repair		
XRCC4	rs6869366	F: 5'-tgaggctcctttccagctctca-3'
	(c.-1746T>G)	R: 5'-agaagcttgtggccgagaagg-3'
LIG4	rs1805388	F: 5'-tggggcctggattgctgggtctg-3'
	(c.26C>T, p.Thr9Ile)	R: 5'-cagcaccactaccacaccctga-3'

of cell proliferation in underground coal miners from Kemerovo Region (Russian Federation) compared to healthy non-exposed men. Our work pointed at that health of coal miners is compromised by high genotoxic stress resulting from exposure to coal residue mixtures containing traces of iron, sulphur, coal ash, heavy metals and PAHs, as well as ionising radiation (28).

In this work, we profiled SNPs in DNA repair genes in 143 genomic DNA samples obtained from coal miners (exposed group) and 127 genomic DNA samples from healthy non-exposed men (control group). The distribution of genotypes and alleles frequencies of the h*OGG1* (rs1052133), *XRCC1* (rs25487), *ADPRT* (rs1136410), *XRCC4* (rs6869366) and *LIG4* (rs1805388) genes in groups is presented in Table 4. There were no significant differences in allele frequencies between exposed and control groups; the distribution of genotypes of the studied genes were in accordance with the Hardy–Weinberg equilibrium in both groups (Table 4). Allele frequencies of h*OGG1* (rs1052133), *ADPRT* (rs1136410) and *LIG4* (rs1805388) genes were similar to their corresponding frequencies in the European population, but for the *XRCC1* (rs25487) and *XRCC4* (rs6869366) genes some deviations were identified. According to the 1000 Genomes project (29), in the European population the frequencies of A and G alleles of the *XRCC1* (rs25487) gene are 37 and 63%, whereas in our experiment these values were 57 and 43% in the exposed group, 59 and 41% in the control group. For the *XRCC4* (rs6869366) gene, the frequencies were 55, 45% (coal miners) and 54, 46% (non-exposed men) for T and G alleles, respectively, as opposed to 94 and 6% in the European population.

All studied the cytogenetic indicators (NDI, BN lymphocytes with MN, NPBs and NBUDs) were tested using ROC analysis to serve as predictors of increased genotoxic risk in coal miners. According to the AUC index, NDI and BN cells with MN were characterised as a good test (AUC = 0.889 and AUC = 8.824), whilst BN cells with NPBs and NBUDs—as a fair test (AUC = 0.762 and AUC = 0.769) (30). As such, NDI and BN lymphocytes with MN can be used for the prediction of genotoxic risk with high sensitivity and specificity.

Based on these results, we then performed a cytogenetic investigation analyzing 1000 BN lymphocytes per each individual enrolled in this study to examine the relationship between different

Table 3. Amplification program for the DNA sample analysis

Temperature, C°	Time	Number of cycles
94	Pause	
93	1 min	1
93	10 s	35
64	10 s	
72	20 s	
72	1 min	1
10	Storage	

Table 4. Distribution of genotype and allele frequencies [*N* (%)] in the studied groups

	Exposed group (*N* = 143)	Non-exposed control group (*N* = 127)	Conformity to the Hardy–Weinberg equilibrium, χ^2 (*P*)	
			Exposed group	Non-exposed control group
h*OGG1*, rs1052133 (c.977C>G, p.Ser326Cys)				
C/C	77 (53.8)	71 (55.9)	0.7636 (0.3822)	0.4324 (0.5108)
C/G	53 (37.1)	46 (36.2)		
G/G	13 (9.1)	10 (7.9)		
C allele frequency	207 (0.72)	188 (0.74)		
G allele frequency	79 (0.28)	66 (0.26)		
XRCC1, rs25487 (c.1196A>G, p.Arg399Gln)				
A/A	48 (33.6)	44 (34.6)	0.1120 (0.7379)	0.1058 (0.7449)
A/G	68 (47.6)	63 (49.6)		
G/G	27 (18.8)	20 (15.8)		
A allele frequency	167 (0.57)	151 (0.59)		
G allele frequency	122 (0.43)	103 (0.41)		
ADPRT, rs1136410 (c.2285T>C, p.Val762Ala)				
T/T	87 (60.8)	76 (59.8)	1.2619 (0.2613)	0.8856 (0.3467)
T/C	46 (32.2)	42 (33.1)		
C/C	10 (7.0)	9 (7.1)		
T allele frequency	220 (0.77)	194 (0.76)		
C allele frequency	66 (0.23)	60 (0.24)		
XRCC4, rs6869366 (c.-1746T>G)				
T/T	41 (28.7)	36 (28.3)	0.7993 (0.3713)	0.2833 (0.5946)
T/G	76 (53.1)	66 (52.0)		
G/G	26 (18.2)	25 (19.7)		
T allele frequency	158 (0.55)	138 (0.54)		
G allele frequency	128 (0.45)	116 (0.46)		
LIG4, rs1805388 (c.26C>T, p.Thr9Ile)				
C/C	71 (49.7)	63 (49.6)	1.5122 (0.2188)	0.3115 (0.5768)
C/T	55 (38.5)	51 (40.2)		
T/T	17 (11.8)	13 (10.2)		
C allele frequency	197 (0.69)	177 (0.70)		
T allele frequency	89 (0.31)	77 (0.30)		

polymorphic variants in the DNA repair genes and cytogenetic indicators (Table 5). Analysis of associations between the SNPs and the level of cytogenetic damage in the exposed group showed that miners with the G/G genotype for the h*OGG1* (rs1052133) gene have a significantly increased ($P < 0.001$ after applying the FDR correction) frequency of BN lymphocytes with MN (13.17‰, 95% CI = 10.78–15.56) in comparison with C/C genotype carriers (10.35‰, 95% CI = 9.59–11.18). In addition, in the exposed group this indicator was significantly increased in carriers of the T/T genotype for the *LIG4* (rs1805388) gene compared to miners harbouring the heterozygous genotype C/T (13.00‰, 95% CI = 10.96–15.04 and 9.69‰, 95% CI = 8.32–11.06, respectively; $P < 0.001$). At the same time, we discovered no significant associations between polymorphisms within the DNA repair genes and cytogenetic abnormalities in non-exposed healthy blood donors.

Finally, using the MDR method, we identified a three-locus model of gene–gene interactions h*OGG1* (rs1052133) × *ADPRT* (rs1136410) × *XRCC4* (rs6869366) associated with higher genotoxic damage in underground coal miners and characterised by cross-validation consistency of 100%, the highest balanced accuracy (the average true-positive and true-negative rates), sensitivity and specificity compared to other models (Table 6). In this model, increased DNA damage was mainly determined by the h*OGG1* (rs1052133) gene (entropy 1.43%); the interactions between the locus h*OGG1* (rs1052133)—*ADPRT* (rs1136410) and h*OGG1* (rs1052133)—*XRCC4* (rs6869366) were characterised by strong synergetic effects (Figure 1). Figure 2 summarises 'protective' and 'risk' allele combinations in the h*OGG1* (rs1052133) × *ADPRT* (rs1136410) × *XRCC4* (rs6869366) model.

Discussion

Genome susceptibility to mutagenic factors is determined by DNA repair efficacy (16). DNA repair is the main protective mechanism against carcinogenic and mutagenic agents (16,31,32). Because the amount of cellular DNA damage directly correlates with the efficacy of DNA repair machinery, it is feasible to search for polymorphic variants in DNA repair genes that can be responsible for increased levels of cytogenetic abnormalities. The importance of such studies is stipulated by the necessity to evaluate individual genotoxic risks as well as risks of developing occupational diseases among workers in order to establish approaches for predicting and preventing such risks in people with different hereditary characteristics.

Underground coal mines are characterised by a wide range of hazards with mutagenic and carcinogenic effects. In particular, environment of these mines is saturated with coal dust, radon (^{222}Rn) and its by-products, PAHs, carbon oxide, phenol, hydrogen sulphide, naphthalene, benzol, etc. Exposure to these agents has been shown to increase carcinogenic and genotoxic risk in coal miners (7).

Here we assessed the relationship between SNPs in DNA repair genes polymorphisms with genotoxic stress in underground coal miners. Healthy males without occupational exposure to genotoxic agents and therefore having a low mutation load were used as a control group. We first calculated genotypes and allele frequencies in the groups and tested whether they conform to the Hardy–Weinberg equilibrium and correspond to allele frequencies in the European population (Table 4). Interestingly, we found that frequencies of *XRCC1* (rs25487) and *XRCC4* (rs6869366) gene alleles showed intermediate values compared to those in Asian and European populations. Allele frequencies of all other SNPs were consistent to their corresponding frequencies in the European population. The differences between exposed and control groups, as well as deviation from the Hardy–Weinberg equilibrium were not detected.

We then examined whether there is a correlation between the presence of certain SNP alleles and the frequency of cytogenetic abnormalities in the groups. We identified that allelic variants in the DNA excision repair (h*OGG1*) and double-strand break repair (*LIG4*) genes are associated with higher MN frequency in miners

Table 5. Association of polymorphisms of the *hOGG1, XRCC1, ADPRT, XRCC4* and *LIG4* genes with the studied cytogenetic indicators and proliferative activity in underground coal miners [mean (95% CI)]

Genotype	Nuclear division index	Micronuclei	Nucleoplasmic bridges	Protrusions (nuclear buds)
h*OGG1*, rs1052133 (c.977C>G, p.Ser326Cys)				
C/C	1.80 (1.76–1.83)	10.35 (9.59–11.18)	3.97 (3.32–4.63)	6.52 (5.40–7.64)
C/G	1.75 (1.71–1.79)	11.72 (10.45–12.98)	3.89 (3.17–4.60)	7.45 (5.77–9.14)
G/G	1.80 (1.70–1.89)	13.17 (11.78–14.56)*	3.67 (1.92–5.41)	7.33 (5.23–9.44)
XRCC1, rs25487 (c.1196A>G, p.Arg399Gln)				
A/A	1.74 (1.69–1.78)	11.23 (10.11–12.35)	4.10 (3.15–5.06)	7.69 (6.06–9.32)
A/G	1.81 (1.77–1.84)	10.93 (9.88–11.97)	3.68 (3.09–4.26)	6.56 (5.25–7.87)
G/G	1.79 (1.74–1.84)	11.31 (9.64–12.97)	4.19 (3.15–5.24)	7.00 (4.98–9.02)
ADPRT, rs1136410 (c.2285T>C, p.Val762Ala)				
T/T	1.79 (1.76–1.82)	11.39 (10.48–12.30)	4.00 (3.44–4.56)	6.97 (5.78–8.15)
T/C	1.77 (1.72–1.81)	10.43 (9.38–11.49)	3.80 (2.92–4.69)	7.61 (6.01–9.21)
C/C	1.78 (1.65–1.91)	11.67 (7.64–15.70)	3.67 (1.33–6.00)	7.56 (6.86–9.25)
XRCC4, rs6869366 (c.-1746T>G)				
T/T	1.80 (1.76–1.84)	11.24 (9.91–12.58)	4.27 (3.42–5.12)	6.32 (4.88–7.75)
T/G	1.77 (1.74–1.81)	10.86 (9.90–11.81)	3.66 (3.10–4.21)	7.22 (5.96–8.49)
G/G	1.77 (1.96–1.84)	11.60 (10.07–13.13)	4.12 (2.57–5.67)	7.56 (4.96–10.16)
LIG4, rs1805388 (c.26C>T, p.Thr9Ile)				
C/C	1.77 (1.74–1.80)	11.21 (10.37–12.05)	3.79 (3.27–4.31)	7.39 (6.23–8.55)
C/T	1.81 (1.76–1.87)	9.69 (8.32–11.06)	3.83 (2.59–5.06)	5.41 (4.87–6.96)
T/T	1.76 (1.69–1.84)	13.00 (10.96–15.04)**	4.81 (3.27–6.36)	7.69 (5.04–10.30)

95% CI, 95% confident interval.

*$P < 0.001$: significant differences in comparison with the C/C genotype.

**$P < 0.001$: significant differences in comparison with the C/T genotype.

Table 6. Characteristics of the model of gene–gene interactions associated with high genotoxic risk in coal miners

	Training balanced accuracy	Testing balanced accuracy	Sensitivity	Specificity	Cross-validation consistency	Precision	Significant test (P)
h*OGG1* (rs1052133) × *ADPRT* (rs1136410) × *XRCC4* (rs6869366)	0.70	0.51	0.47	0.55	10/10	0.63	0.0001
h*OGG1* (rs1052133) × *ADPRT* (rs1136410) × *XRCC1* (rs25487)	0.61	0.50	0.40	0.51	8/10	0.56	0.01
XRCC1 (rs25487) × *XRCC4* (rs6869366) × *LIG4* (rs1805388)	0.63	0.41	0.43	0.49	7/10	0.48	0.01

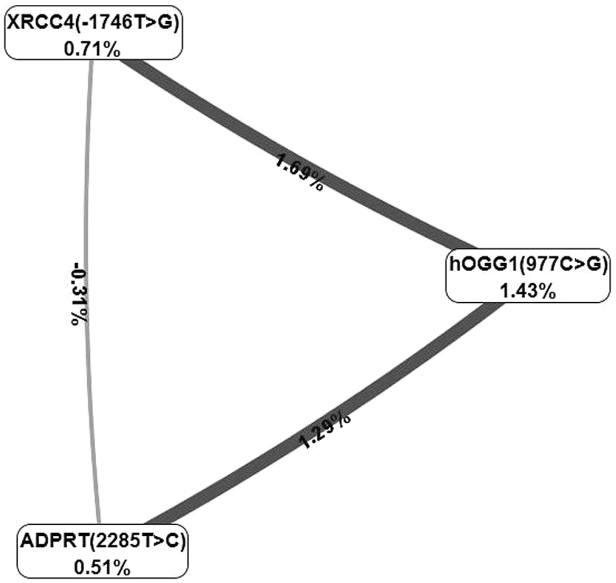

Figure 1. Entropy-based radial graph of gene–gene interactions in coal miners. Entropy values in cells reflect independent effects of indicated allelic variants whereas those in connecting lines represent the effect of interaction. The dark-grey lines reflect a high degree of synergy whilst the light-grey line indicates a redundancy.

but not in unexposed men. The main mechanism of MN formation is the misrepair of double-strand breaks. In this case unrepaired DNA damage results in the formation chromatid and chromosome fragments which are eventually being enclosed by a nuclear membrane in anaphase and transformed to MN (18,23,33–35). Other potential mechanism of MN formation is simultaneous excision repair of damaged or inappropriate bases incorporated into DNA that are in proximity and located on opposite DNA strands (18,36,37).

The human *OGG1* (h*OGG1*) (8-oxoguanine glycosylase 1) gene is located on chromosome 3p26 and encodes two isoenzymes, α-hOGG1 and β-hOGG1. These enzymes play an important role in cell protection against oxidative DNA damage; in particular, they catalyze the cleavage of the N-glycosidic bond between the aberrant base and the sugar-phosphate backbone generating an apurinic (AP) site. Next, the phosphodiester bound 3′ from the AP site is cleaved by an elimination reaction, leaving a 3′-terminal unsaturated sugar and a product with a terminal 5′phosphate (38). The G/G genotype of the h*OGG1* gene 977C>G polymorphism was reported to decrease protein activity resulting in impaired DNA repair (39) and was associated with increased cancer risk (40–44). At the same time, the C/C genotype of this SNP correlated with the synthesis of active enzyme resulting in a more effective excision of 8-oxoguanine and decreased mutation load (45,46). Importantly, our results are concordant with other reports. Increased MN frequency in lymphocytes were observed in individuals exposed to ionising radiation and carrying C/G and G/G genotypes for the h*OGG1* gene 977C>G polymorphism compared to the C/C genotype (47). Similar association was reported for carriers of the G allele and mutation load in male workers exposed to heavy metals (48). It is known that ionising radiation can have not only direct (ionisation of DNA molecule, release of energy, excitation of valent electrons and, as a result, rupture of chemical bounds and damage in intact structure of DNA), but also indirect effects (ROS generation as a result of water radiolysis), so such proteins like hOGG1 eliminating oxidative DNA damage play important role in cell protection in conditions of ionising radiation exposure (49,50). Therefore, we can explain our results about the association of h*OGG1* (rs1052133) polymorphism and DNA damage in coal miners by oxidative stress and ROS action not only due inorganic substances and PAHs inhalation and inflammation, but also exposure to ionising radiation from different sources (gamma radiation, radon and its decay products). At the same time, some authors reported no significant differences between DNA damage and the h*OGG1* gene polymorphism (51,52). This discrepancy may be due to, on the one hand, insufficient knowledge of molecular and genetic mechanisms of individual susceptibility to oxidative stress in individuals exposed to different harmful factors; and on the other hand, the complex nature of genotoxic stress deriving from multiple sources and different mechanisms of response to it. The *LIG4* gene is one of the key genes involved in the non-homologous end joining double-strand breaks repair. Lig4 protein is a DNA ligase which uses ATP for adenylation and then transfers the AMP group at the 5′-end of DNA chain. Thus, the hydroxyl group of 3′-end of the opposite DNA strand undergoes nucleophilic attack that liberates AMP and promotes the formation of the ligation product. The mutation in *LIG4* (rs1805388) gene leads to a non-synonymous substitution of Thr to Ile at the N-terminal region of Lig4 protein and is essential for its functionality (53). It was reported to reduce adenylation and activity of ligation by 2- or 3-fold and increases the hydrophobicity of this protein (54,55). Changes in the structure of Lig4 protein lead to disruption of its interaction with XRCC4 protein, thereby reducing the efficiency of DNA repair. The T allele homozygotes for this SNP exhibited a defective DNA repair (53) and correlated with higher chromosome aberration (CAs) frequency in lymphocytes (56). This SNP was also linked to immunodeficiency (57,58), radiation pneumonitis (59) non-small cell lung cancer (60) and glioma (61). In keeping with these observations, here we report a decrease in the efficiency of DNA repair in coal miners with the T/T genotype for this SNP, which is reflected in the increased frequency of MN in BN lymphocytes. The non-homologous end joining is an important mechanism eliminating the most dangerous form of DNA damage, namely DNA double-strand breaks, which were reported to be induced by ionising radiation (49), confirming this factor play important role in genotoxic load in coal miners.

Thus, our results demonstrate that upon the exposure to genotoxic factors, coal mine workers carrying 'risk' genotypes associated

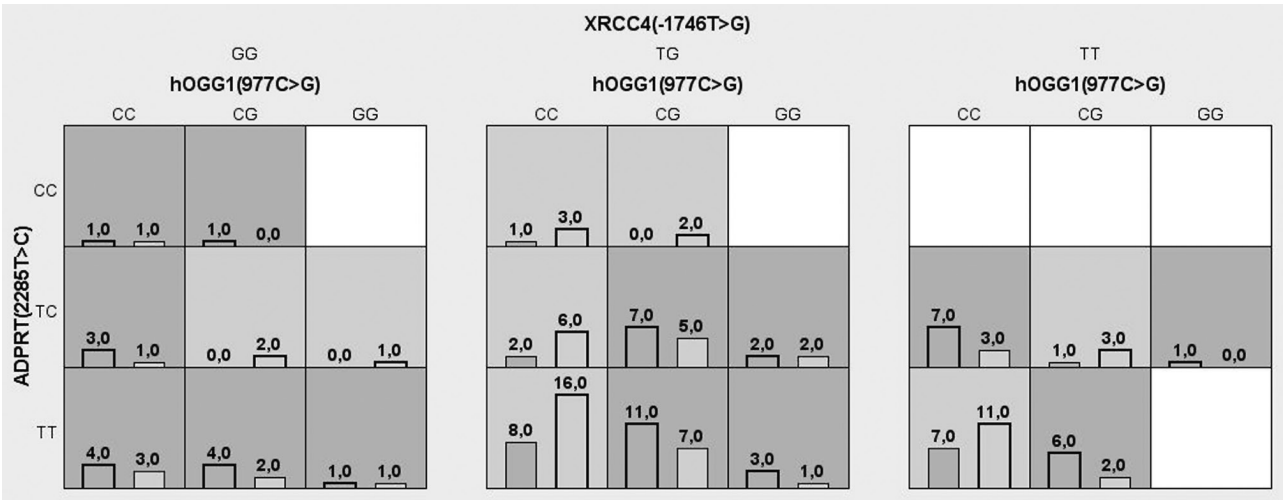

Figure 2. Allele combinations of indicated SNPs associated with high (dark-grey cells) and low (light-grey cells) genotoxic risk in coal miners.

with defective DNA repair exhibit higher levels of cytogenetic damage. Importantly, there was no correlation between the cytogenetic status and SNPs of DNA repair genes in non-exposed healthy men, further confirming the reliability of our results.

Finally, we for the first time determined the three-locus model of gene-gene interactions associated with the increased genome instability in coal miners (Figure 1). Figure 2 demonstrates the 'risk' (dark-grey cells) and 'protective' (light-grey cells) alleles combinations. It is interesting that separately polymorphisms in *ADPRT* (rs1136410) and *XRCC4* (rs6869366) genes have no effects on DNA damage in coal miners, but in the combined model with the h*OGG1* (rs1052133) SNP they show a significant increase of DNA damage in carriers of certain alleles combinations. We therefore propose these genotype combinations as key players in modulating the genotoxic risk in people working in hazardous conditions.

Conclusions

Here we demonstrate that underground coal miners with the G/G genotype for the h*OGG1* (rs1052133) gene and carriers of the T/T genotype for the *LIG4* (rs1805388) gene have an increased individual susceptibility to the mutagenic and genotoxic exposure in coal mining conditions. The three-locus model of gene–gene interactions h*OGG1* (rs1052133) × *ADPRT* (rs1136410) × *XRCC4* (rs6869366) is associated with higher genotoxic risk in coal miners. These SNPs and their combinations may be used as molecular predictors of occupational risks in underground coal mines.

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