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Genetic Predisposition to Chronic Dust Bronchitis among Potash Miners

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ABSTRACT

Chronic respiratory diseases represent a prevalent occupational disease worldwide. It is suggested that polymorphisms in genes of enzymes of monooxygenase (microsomal epoxide hydrolase 1 (EPHX1), cytochromes (CYPs)) and antioxidant glutathione S-transferases (GSTs) systems might potentiate susceptibility to respiratory system pathology in workers employed in mining. There are a large number of studies on genetic markers for occupational pulmonary diseases, but the majority of them concerns workers of the coal industry. Therefore, we aimed to investigate genetic predisposition to chronic dust occupational bronchitis (CB) among potash miners. The study population consisted of 38 males with CB and 18 controls. Deletions in *GSTM1* and *GSTT1* genes were analysed using multiplex PCR. Polymorphisms of *CYP1A1* (Ile462Val), *EPHX1* (His139Arg) and *GSTP1* (Ile105Val) were detected using PCR followed by restriction fragment length polymorphism (PCR-RFLP) analysis. Significantly increased risk for CB was observed for subjects with polymorphism in exon5 (Ile105Val) of *GSTP1* (OR=8.6, χ^2=8.794, *p*=0.001) and polymorphism in exon4 (His139Arg) of *EPHX1* (OR=8.6, χ^2=4.4, *p*=0.04). This study showed that the *GSTP1* (Ile105Val) and *EPHX1* (His139Arg) polymorphisms are closely associated with the increased risk for chronic dust occupational bronchitis among potash miners.

Key words: Bronchitis, Genetic polymorphism, Potash miners, Predisposition.

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) represents a leading cause of death worldwide. As much as 15% of COPD cases have been attributed to occupational exposures (Balmes et al., 2003; Blanc 2012). COPD has serious medical, social and economic consequences as the clinical signs of disease develop slowly, so complicating early diagnosis and often results in pulmonary and extrapulmonary complications (Eisner et al., 2011). The risk of COPD is high in industries where workers operate in the atmosphere of industrial aerosols. As a result such a condition is typical for underground workers who are exposed to coal or mineral dust caused by mining activities. So, chronic dust bronchitis is one of the widespread occupational forms of COPD. It is known that in addition to exogenic influences (such as components and duration of industrial aerosol impact, smoking, concurrent infections and associated diseases) individual features including genetic factors may play roles in the pathogenesis of respiratory diseases (Blanc and Toren, 2007; Omland et al., 2014). Currently the

majority of genetic studies of occupational pulmonary diseases concerns workers of the coal industry (Cohen et al., 2008; Tomas and Linus, 2011).

One of the principal molecular mechanisms of chronic respiratory diseases development is an oxidative stress which results from inflammatory processes in respiratory tract due to long contact with industrial pollutants. Therefore, enzymes of monooxygenase EPHX1, CYPs and antioxidant GSTs systems that carry out effective protection of cells against oxidative damages and xenobiotics, play an essential role in defense responses of a pulmonary tissue. It is known that chronic bronchitis among potash miners is polygenic disease. *GSTs, CYP1A1* and *EPHX1* are genetically polymorphic. Variations in these genes were previously suggested to be associated with high risk of COPD development. The current data on the genetics of COPD was highlighted in the recent reviews (Bossé 2012; Wu et al., 2014). The conditions in which potash miners operate are unique because of the ambivalent effect of industrial mineral sylvinite dust and speleomicroclimate. In spite of the evidence of positive influence of speleotherapy on immune and endocrine status, prolonged exposure to industrial aerosol among potash mines is clinically approved to lead to the development of occupational pathology. Therefore, early diagnostics of respiratory pathology among potash miners is crucial. Thus, the aim of this work was to study genetic predisposition to chronic dust occupational bronchitis among potash miners.

MATERIALS AND METHODS

Subjects

We randomly assigned every second sick potash miner of Joint Stock Company Belaruskali (Soligorsk, Belarus) to achieve 38 males in the CB group. Control group consisted of 18 healthy workers. The difference in age, underground experience and tobacco consumption between the control subjects and CB patients was not significant ($p>0.05$) (Table 3). Participants signed a written informed consent to pass a spirometry and to donate blood for genotyping, completed a modified version of the 1978 ATS-DLD Epidemiology Questionnaire (Ferns, 1978).

Pulmonary function tests

Spirometry was performed using Electronic desktop spirometer (MAS-1, Belarus) in accordance with ERS guidelines (GOLD, 2010). Subjects were asked to avoid bronchodilator medication use for at least 4 h prior to spirometry. The manipulations were performed in a standardized manner with subject seated and wearing a nose-clip. Chronic bronchitis was defined according to affirmative responses to questionnaire items for both chronic cough and chronic phlegm production for at least 3 months per year for at least 2 years and/or obstructive spirometry abnormalities in conformity with the global initiative for COPD (GOLD, 2013). Exclusion criteria included acute CB exacerbation during inclusion, asthma, allergies, and severe chronic disease from clinical significance according to the investigators judgment, other pulmonary diseases such as diffuse diseases of the lung parenchyma, as well as severe diseases such as cancer, instable disease condition other than CB (for example, coronary heart disease, chronic heart failure, kidney failure, liver diseases, infection diseases including acute tuberculosis, or immunodeficiency).

Isolation of DNA

Whole peripheral blood samples were obtained by phlebotomy of an antecubital vein. Samples were anticoagulated with ethylene diamine tetra-acetic acid (K_3-EDTA) and stored at –20°C until DNA extraction. Genomic DNA was extracted from 500 µL whole blood using the Nucleospin Tissue kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. DNA concentrations were determined by NanoDrop 2000c (Thermo Scientific, USA). For genotype *CYP1A1*, *EPHX1* and *GSTP1* polymorphisms, genomic DNA was amplified by the method of PCR using amplificator MJ Mini^{Tm} Personal Thermal Cycler (Bio-Rad Laboratories, USA). PCR assays were performed using the primer pairs listed in Table 1. Genomic DNA (50 ng) was added to the PCR mixture containing 12 pmol µl^{-1} of each primer, 500 µM dNTP mixture, 1.5 mM $MgCl_2$, 1.5 units of *Taq*-polymerase (Fermentas, Lithuania) and sterile water purified of nucleases to the final volume of 40 µl. In case of *GSTM1*, *GSTT1* null genotypes determination multiplex PCR was performed with co-amplification of the albumin gene as an internal control in 50 µl PCR mixture consisting of 100 ng of genomic DNA, 1.5 mM $MgCl_2$, 15 pmol µl^{-1} of *GSTM1* and *GSTT1* primers, 7.5 pmol µl^{-1} of albumin primers, 600 µM dNTP mixture, 1.5 units of *Taq*-polymerase (Fermentas, Lithuania). The PCR temperature conditions are presented in Table 2.

Genotyping protocol

The PCR products were analysed in 1.8% agarose gel stained with DNA-dye Zubr Green I (PrimeTech, Belarus) and detected by UV-illumination using image-system VersaDoc MP 4000 (Bio-Rad Laboratories, USA). For RFLP analysis each PCR product was restricted with 10 units of corresponding restrictase (Fermentas, Lithuania) (Table 1) for 4 h at 37ºC and resolved using 1.8% agarose gel. Deletions of *GSTM1* and *GSTT1*

Table 1. PCR primers and conditions of RFLP analysis for gene polymorphism screening.

Gene, localization	Polymorphism	Primers, (5´→3´)	Product size, bp	Restrictase	Alleles, bp
CYP1A1 15q22-24	7th exon A4889G (Ile462Val)	F: GAAAGACCTCCCAGCGGTCA R: GAACTGCCACTTCAGCTGTCT	187	*Hinc* II	A (48, 139), G (19, 48, 120)
GSTM1 1q13.3	Deletion	F: GAACTCCCTGAAAAGCTAAAGC R: GTTGGGCTCAAATATACGGTGG	219	-	Norm (219), Deletion (0)
GSTT1 22q11.2	Deletion	F: TCACCGGATCATGGCCAGCA R: TTCCTTACTGGTCCTCACATCTC	459	-	Norm (459), Deletion (0)
GSTP1 11q13	5th exon A313G (Ile105Val)	F: ACCCCAGGGCTCTATGGGAA R: GAGGGCACAAGAAGCCCCT	175	*Alw 26*I	A (175), G (82, 93)
EPHX1 1g42.1	4th exon A415G (His139Arg)	F: ACATCCACTTCATCCACGT R: ATGCCTCTGAGAAGCCAT	210	*Rsa* I	A (210), G (47, 163)

Table 2. PCR temperature conditions for gene polymorphism screening.

Gene	PCR temperature conditions
CYP1A1	95°C – 5 min, (94°C – 30 s, 56°C – 30 s, 72°C – 30 s) x 35 cycles, 72°C – 5 min
GSTT1	95°C – 5 min, (95°C – 40 s, 64°C – 1 min, 72°C – 1 min) x 35 cycles, 72°C – 5 min
GSTM1	
Albumin	
GATPI	95°C – 5 min, (94°C – 30 s, 57°C – 40 s, 72°C – 40 s) x 35 cycles, 72°C – 5 min
EPHX1	95°C – 5 min, (95°C – 40 s, 60°C – 40 s, 72°C – 40 s) x 35 cycles, 72°C – 5 min

genes were analyzed after multiplex PCR amplification. In both *GSTM1* and *GSTT1*, polymorphisms represent gene deletions which are known to be responsible for the existence of null alleles. PCR produced DNA fragments corresponding to 459 bp (*GSTT1*), 219 bp (*GSTM1*) and 350 bp (albumin). Subjects with null homozygous genotype did not show the respective amplified DNA fragment in the agarose gel.

In order to detect *CYP1A1*, *EPHX1* and *GSTP1* polymorphisms, RFLP analysis was performed. *CYP1A1*, *EPHX1* and *GSTP1* were detected based on the relative size of the observed restriction fragments. In the analysis of *GSTP1* genotype endonuclease *Alw 26*I was used to reveal the polymorphism in exon5 (Ile105Val). DNA samples from wild-type homozygotes (AA) were expected to produce 175 bp band, DNA samples from mutant (GG) homozygotes yielded 82 bp and 93 bp bands, whereas DNA samples from heterozygotes (AG) contained all three bands 175 bp, 93 bp and 82 bp. In the analysis of *CYP1A1* genotype endonuclease *Hinc* II was used to reveal the polymorphism in exon7 (Ile462Val). DNA samples from wild-type homozygotes (AA) were expected to produce 48 bp and 139 bp bands, DNA samples from mutant (GG) homozygotes yielded 19 bp, 48 bp and 120 bp bands, whereas DNA samples from heterozygotes (AG) contained all four bands 19 bp, 48 bp, 120 bp and 139 bp. In the analysis of *EPHX1* genotype, endonuclease *Rsa* I was used to reveal the polymorphism in exon4 (His139Arg). DNA samples from wild-type homozygotes (AA) were expected to produce 210 bp band, DNA samples from mutant (GG) homozygotes yielded 47 bp and 163 bp bands, whereas DNA samples from heterozygotes (AG) contained all three bands of 47 bp, 163 bp and 210 bp.

Statistical analysis

Data expressed as percentage (%) or mean ± standard deviation. Group comparisons were performed by the Mann–Whitney U-test (Statistica 6.0, StatSoft, Inc., USA). Association of the genotype polymorphisms between CB patients and healthy subjects were analyzed using the χ^2 test for 2x2 tables. The risk imparted by a particular phenotype was calculated using the odds ratio (OR) equations. The OR is given by: (A/B)/(C/D), where A and B are the number of patients with and without the risk allele, respectively, and C and D are the number of controls with and without the risk allele respectively. *P*-values < 0.05 were considered significant.

RESULTS AND DISCUSSION

Table 3 shows the characteristics of the 56 study

Table 3. Characteristics of individuals in group of chronic bronchitis (CB) and control group.

	CB group	Control group	*P*-value
Subjects, n	38	18	
Age, years	41.2±9.8	39.6±9.9	0.21
Underground experience, years	16.3±10.4	13.9±10.0	0.13
Smoking pack-years median (range)	14.5 (6-28)	14.0 (4-40)	0.17
Current smokers, %	44.7	44.4	0.98
FEV_1/FVC, %	72.1±8.6	86.2±1.6	<0.001

Data are presented as mean±SD, unless otherwise stated.
FEV_1 – forced expiratory volume in one second; FVC – forced vital capacity.

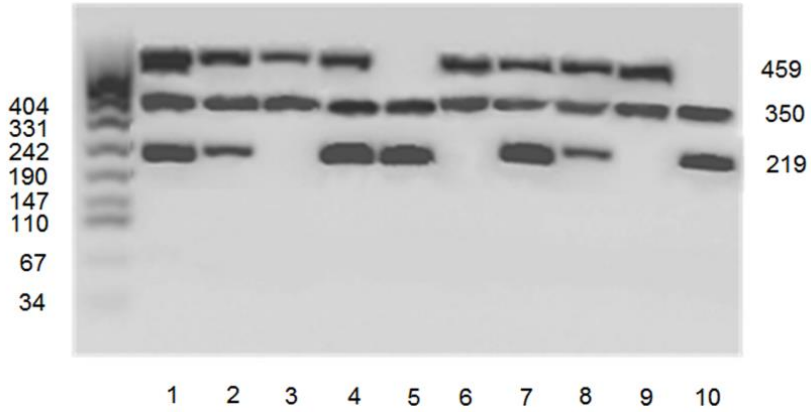

Figure 1. Electrophoresis of multiplex PCR amplification of *GSTM1* (219 bp), *GSTT1* (450 bp) and *albumin* as an internal control (350 bp). Lanes 1, 2, 4, 7, 8 – wild genotype of *GSTM1*(+)/wild genotype of *GSTT1*(+), lanes 3, 6, 9 – null genotype of *GSTM1*(-)/wild genotype of *GSTT1*(+); lanes 5, 10 – wild type genotype of *GSTM1*(+)/null genotype of *GSTT1*(-). DNA markers are shown on the left. Sizes of PCR products (bp) are indicated on the right.

subjects. The difference in age and tobacco consumption between CB patients and the control subjects was not significant (p>0.05). The mean of ratio of forced expiratory volume in one second (FEV_1) to forced vital capacity (FVC) of the CB group was approximately 16.3% lower than in the control group (p<0.001). The results of genetic polymorphisms of *GSTs* genes showed differences between the studied groups, but not significant for all genes. Frequency of *GSTM* (-) in the CB group was 47.4% (18 persons from 38) whereas in the control group – only 27.8% (OR=2.3; χ^2=1.94, p=0.16) (Table 4 and Figure 1). There was not significant association between the *GSTT1* polymorphic (null) genotype and the risk of occupational CB. A null *GSTT1* genotype was observed in 26.3% subjects of the CB group against 11.1% in the control group (OR=2.9; χ^2=1.68, p=0.2) (Table 4 and Figure 1). Significant differences were found following the analysis of polymorphic A313G locus in exon5 of *GSTP1* which is known to be responsible for the enzyme substrate specificity (Table 4 and Figure 2a). Substantial frequency increase of *GSTP1* polymorphic (mutant) variant allele G was revealed in 40.8% subjects of the CB group against 8.3% in the control group (OR=8.6, χ^2=8.794, p=0.001) (Table 4). Thus, wild type frequencies of *GST* genes responsible for expression of *GSTs* of all studied classes (μ, π and τ) were increased in the CB group.

The relative frequencies of polymorphism *CYP1A1* (Ile462Val) did not differ between CB and control groups. The analysis revealed only one heterozygotic AG genotype in the control group (5.6%) and one homozygous mutant GG genotype in the CB group (2.6%) (OR=0.5, χ^2=0.3, p=0.58) (Table 4 and Figure 2b). All other genotypes of individuals on this locus corresponded to homozygous wild AA type (Table 4 and Figure 2b). Genetic polymorphism in *EPHX1* (His139Arg) showed that the CB patients had significant differences in frequency of mutant G allele as compared with control group (42.1% versus 27.8%, OR=3.4, χ^2=4.4, p=0.04) (Table 4 and fig. 2c). Despite lacking differences on distribution of GG mutant homozygotes (15.8% in the CB group and 16.7% in the control group, OR=0.9, χ^2=0.02, p=0.9), AG heterozygotic genotype was found to prevail in the CB group (52.6%) against 22.2% in the control

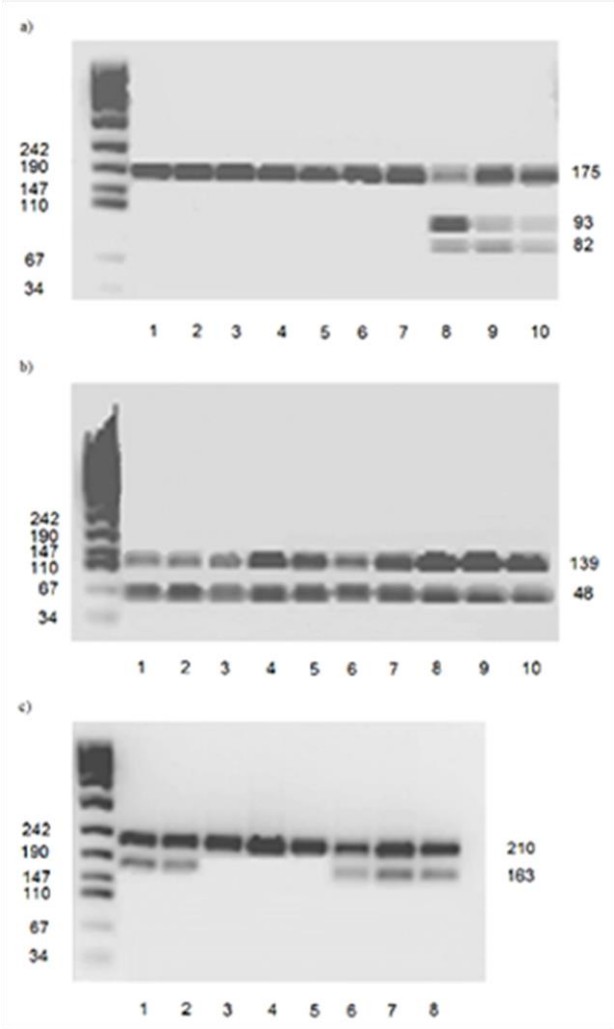

Figure 2. Electrophoresis of PCR and enzymatic restriction products **(A)** of exon5 of *GSTP1* after restriction by *Alw 26*I (lanes 1–7 show amplification products of wild homozygous genotype AA, lanes 8–10 show amplification products of heterozygous genotype AG), **(B)** of exon7 of *CYP1A1* restriction by *Hinc* II (lanes 1–10 show amplification products of wild homozygous genotype AA) and **(C)** of exon4 of *EPHX1* after restriction by *Rsa* I (lanes 3–5 show amplification products of wild homozygous genotype AA, lanes 1, 2, 6–8 show amplification products of heterozygous genotype AG). DNA markers are shown on the left. Sizes of PCR products (bp) are indicated on the right.

group, p=0.031 (table 4 Figure and 2c). Interestingly, it was found that CB patients had at least one mutation among investigated genes. There were polymorphisms in two and more genes in 27 CB patients (71%) whereas 3 (16.7%) of control individuals did not have mutations in all studied polymorphic loci, 13 (72.2%) of control subjects displayed polymorphism only in one gene and 2 (11.1%, p<0.001 in comparison with CB) of control subjects had polymorphisms in two and more genes.

Genetic polymorphisms in genes of monooxygenase and antioxidant enzymes might affect the function of proteins and significantly induce the detoxification and reduce oxidative stress, thereby playing a crucial role in CB development. Our data suggest that *GSTT1, GSTM1, GSTP1* and *EPHX1* genotypes influence predisposition to chronic occupational dust bronchitis. GSTs represent a superfamily of enzymes involved in the metabolism of electrophilic substances responsible for detoxification by conjugation with glutathione. In addition, they have antioxidant capacities and can affect the systemic and

local oxidative stress levels (Strange at al., 2000; Wu et al., 2014). The high degree of *GST* genetic polymorphisms has a significant impact on synthesis, function and activity of enzymes leading to variations in individual ability in xenobiotics biotransformation and antioxidant protection. Polymorphisms of *GSTT1* and *GSTM1* are related to gene deletion resulting in the loss of enzyme activity. Increasing frequency of null allele of *GSTT1* was shown to be associated with bronchial asthma (Kabesch at al., 2004; Minelli at al., 2010).

It was reported that *GSTM1* null genotype frequency increased in COPD (Lakhdar at al., 2010), chronic bronchitis (Baranova at al., 1997), arteriosclerosis (Pessah-Ramussen at al., 1992), emphysema (Harrison at al., 1997), lung cancer (Harrison at al., 1997; Seidegard at al., 1990), bladder cancer (Bell at al., 1993; Brockmuller at al., 1994), adenocarcinoma (Strange at al., 1991), and colorectal cancer (Zhong at al., 1993b). Genetic polymorphism of *GSTP1* results from transition of G to A in exon 5, which causes Ile 105 to Val 105 mutation. As a result GSTP1 enzyme efficiency changes. Associations of *GSTP1* polymorphism with COPD and bronchial asthma were also previously reported in (Minelli et al., 2010; Tamer et al., 2004). Nevertheless, in the work of Cheng et al. (2004) no differences were observed in the frequency of polymorphic genotypes for *GSTP1* and *GSTT1* in patients with COPD and control subjects. Yucesoy et al. (2005) have shown no significant differences in distribution of null genotypes between patients and healthy controls in the study of antioxidants genes association with progressive fibrosis in mine workers. Yechshzhanov et al. (2011) demonstrated that *GSTM1* and *GSTT* null alleles are associated with predisposition to COPD and they do not play a substantial role in susceptibility to bronchial asthma. Contradictory data about role of *GSTM1, GSTT1* and *GSTP1* polymorphisms in pathogenetic mechanisms of pulmonary disorders may result from population frequencies of *GST* alleles distributions and/or respiratory impairment etiology. For example, the frequency of the *GSTT1* null genotype was highest among Chinese (64.4%), followed by Koreans (60.2%), African-Americans (21.8%) and Caucasians (20.4%), whereas the frequency was lowest among Mexican-Americans (9.7%) (Nelson et al., 1995). *GSTM1* null genotype in turn is presented from 38 to 54% of the European population and from 33 to 63% of the Asian populations (Saadat et al., 2001).

In our work the high degree of association for CB development was identified for polymorphic loci of *GSTP1* and *EPHX1*. It should be noted that among all *GST* genes only *GSTP1* is expressed in alveoli, alveolar macrophages and bronchioles (Cantlay et al., 1994). That explains our data about *GSTP1* role in sensitivity of lungs to sylvinite aerosol action. CYP1 as well as other members of cytochromes P450 family participates in metabolism of alien chemicals, promoting transformation of xenobiotics into polar hydrophilic substances for their further removal. Akhmadishina et al. (2005) have shown that heterozygotic AG genotype of *CYP1A1* displayed higher frequency in workers of chemical manufactures, coal, iron or copper-zinc mines, with toxic or dust bronchitis. Our results did not reveal association of *CYP1A1* polymorphism with chronic bronchitis among potash miners. Polymorphism was observed only in one allele of A4889G *CYP1A1* locus while the second had wild type (Table 4). Besides cytochromes, EPHX1 is known to be an important component of microsomal hydroxylation systems of lungs and also provides xenobiotics detoxification in the first-pass biotransformation (Cheng et al., 2004). Histidine is replaced by arginine in 139 position (His139Arg) resulting in functional polymorphism in exon4 (A415G) of *EPHX1*. This polymorphism increases enzyme activity approximately by 25% (ultrarapid metabolizer allele) (Hassett et al., 1994). The accelerated transformation of epoxide derivatives to highly active metabolites leads to DNA damage that promotes increase in quantity of chromosomal aberrations and causes development of lung neoplasm (Omiecinski et al., 2000). It was demonstrated that *EPHX1* mutations were associated with lung dysfunction (Bossé 2012; Sandford et al., 2001). In our work we revealed that the differences in the frequencies of *EPHX1* (His139Arg) polymorphism between CB and control groups were statistically significant ($p<0.05$). Consequently, *EPHX1* polymorphism may be associated with an increased risk for developing of the chronic bronchitis among potash miners. This results correspond to the data reported an association of *EPHX1* genotype with lung function impairment (Bossé 2012; Sandford et al., 2001). Our data demonstrated that the presence of a polymorphism in two or more studied genes is a major risk factor of occupational pathology of the respiratory system among potash miners. The combination of genetic variants including at least two mutant genotypes of monooxygenase and antioxidant systems might be significant indicators of susceptibility to chronic dust bronchitis among potash miners (OR=19.6, $\chi^2=17.6$, $p<0.01$).

CONCLUSION

The given study showed that the *GSTP1* (Ile105Val) and *EPHX1* (His139Arg) polymorphisms are closely associated with the increased risk for chronic dust occupational bronchitis among potash miners. Thereby, the use of genotyping data allow to form groups with high risk of chronic dust occupational bronchopulmonary pathology development among potash miners who need personal preventive medical care that undoubtedly preventing disability and preserving quality of life.

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