Praca oryginalna *Original paper*

TP53 gene polymorphisms with mammary gland tumors and aging in bitches[1)]

BARTOSZ KEMPISTY*, **, KATARZYNA ZAORSKA*, DOROTA BUKOWSKA***, SYLWIA CIESIÓŁKA*, KATARZYNA WOJTANOWICZ-MARKIEWICZ***, MARCIN NOWAK****, PAWEŁ ANTOSIK***, MAREK GEHRKE***, KLAUS-PETER BRÜSSOW*****, MAŁGORZATA BRUSKA**, MICHAŁ NOWICKI*, MACIEJ ZABEL******

*Department of Histology and Embryology, **Department of Anatomy, Medicine Faculty I, Poznan University of Medical Sciences, Swiecickiego 6 St., 60-781 Poznan, Poland
***Institute of Veterinary Sciences, Faculty of Animal Breeding and Biology, Poznan University of Life Sciences, Wolynska 35 St, 60-637 Poznan, Poland
****Department of Pathology, Faculty of Veterinary Medicine, Wroclaw University of Life Sciences, C. K. Norwida 31 St., 50-375 Wrocław, Poland
*****Department of Reproductive Biology, Leibniz Institute for Farm Animal Biology, Dummerstorf, Germany
******Department of Histology and Embryology, Wroclaw Medical University, 6a Chalubinskiego St., 50-368 Wroclaw, Poland

Received 15.05.2014 *Accepted 30.10.2014*

Kempisty B., Zaorska K., Bukowska D., Ciesiółka S., Wojtanowicz-Markiewicz K., Nowak M., Antosik P., Gehrke M., Brüssow K.-P., Bruska M., Nowicki M., Zabel M.

TP53 gene polymorphisms with mammary gland tumors and aging in bitches

Summary

Blood samples were collected from 22 bitches with diagnosed mammary gland malignant tumors, according to the WHO's International Classification of Disease for Oncology System. The control group (n = 35) consisted of geriatric (> 5 to 10 years old), mature (2-5) and young (1-2) females. We found two novel single nucleotide variants in the coding region of exon 3 and in intron 7 of gene TP53. We did not observe statistical significance in allele or genotype distribution between the tumor and control groups. However, the prevalence of allele C (Odds ratio = 3.0; p = 0.2024) similarly to genotype CT (Odds ratio = 3.3; p = 0.1783) was higher in the control group than in the investigated cancer group. Nonetheless, additional studies on larger groups of canines are required to confirm the presented results.

Keywords: mammary glands, malignant tumor, bitch, aging, polymorphism TP53

In recent years, an increased number of different tumor types have been diagnosed in domestic bitches, and cancer is the most frequent cause of mortality and morbidity in female dogs (10). The mammary gland tumor is the most frequent one diagnosed in domestic bitches. Histopathological characteristics of canine mammary tumors (CMT) are well defined; however, the knowledge on the molecular carcinogenesis of CMT is far from complete, although significant similarities and also differences exist between canine and human mammary tumors at the molecular level (8). Currently, several techniques have been developed to define the canine genome map and new molecular markers for CMT induction, invasion, and/or progression (15). In our study we describe the application of DNA sequencing analysis to identify mutations and/or polymorphisms in the *TP53* gene in domestic bitches with mammary tumors.

TP53 belongs to the transcription factors that regulate the target gene expression profile and induce cell arrest, apoptosis, cell senescence, as well as DNA repair and cell metabolism. Moreover, TP53 is one of the proteins involved in the induction of cell apoptosis through a non-transcriptional cytoplasmic process. In unstressed cells, p53 is kept inactive through the actions of the ubiquitin ligase MDM2 as well as via mutations of the *TP53* gene itself. Furthermore, the inactivation may be associated with loss of cell signaling upstream or downstream of p53 (2, 19, 21).

Since the activity of TP53 is associated with cells senescence, the incidence of mutations in female dogs may be related to body aging as well as tumor

[1)] The study is supported by the Polish Ministry of Scientific Research and Higher Education (Grant No. 5279/B/P01/2011/40).

genesis. *TP53* gene mutations and polymorphisms in canines were recognized in transmissible venereal tumors, osteosarcoma and brain tumors (7, 16, 18, 24). Although an association of *TP53* gene polymorphisms and the incidence of human cancer has been described, the link between canine *TP53* gene polymorphisms and the incidence of mammary gland tumor remains poorly recognized (23).

Therefore, the aim of the present study was to identify *TP53* gene mutations/polymorphisms in bitches of different ages and with diagnosed mammary gland malignant tumor.

Material and methods

Animals and sample collection. We analyzed causes of "neoplasmatic diseases" in 22 bitches with malignant mammary tumors, obtained during surgery in the Small Animal Clinic at the University of Live Sciences, Poznan, Poland. The tumors were diagnosed by histological examination. Representative portions of tissue samples were fixed in 10% buffered formalin and then dehydrated and embedded in paraffin. In HE sections, tumors were classified according to the WHO criteria for canine mammary neoplasms (13) and coded according to the WHO International Classification of Disease for Oncology System (ICD-O). All of the tissue samples were closely examined by a pathologist and recognized as simple, complex or special type of carcinoma. Additionally, whole peripheral blood samples were taken from all 22 oncological patients and 36 bitches free of any type of cancer (served as controls). The blood was taken from the cephalic vein during routine procedures in the clinic, collected in vials with EDTA and frozen in –80°C until further molecular analyses. Controls were divided into three subgroups differing in age according to the classification proposed by Jugdutt et al. (7): geriatric (> 5 to 10 years old, M 8,1, SD 1,8; n = 15), mature adult (> 2 to 5 years old, M 3,3, SD 0,8; n = 10) and young (1 to 2 years old, M 1,5, SD 0,3; n = 11) bitches.

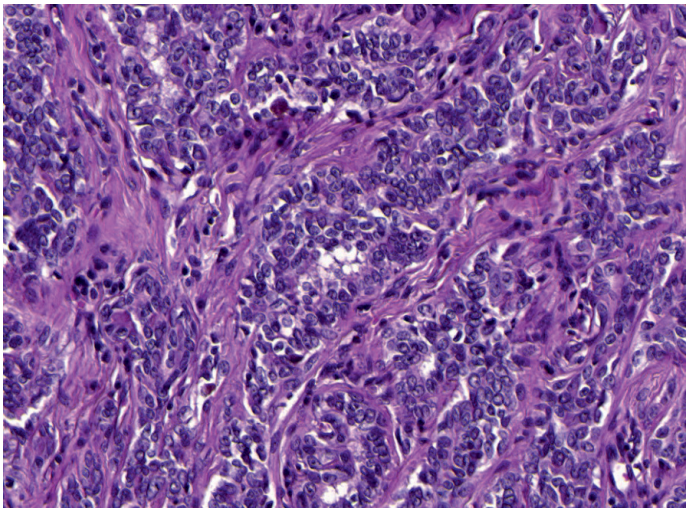

Fig. 1. Histochemical analysis of adenocarcinoma in bitches
Explanation: The histochemical slides were performed after routine procedure of HE. The blood samples were collected from 22 bitches with diagnosed adenocarcinoma. All of tissue samples were strictly examined by pathologist.

Tab. 1. Polymerase chain reaction conditions for the *TP53* gene

Amplified gene region	Primer sequence	Primer annealing conditions
exon 2	Forward 5'-GGAAGCAAACCAGCACTGAC-3' Reverse 5'-GGACCGAGAGGAGTCTCAAA-3'	59°C for 30 sec
exon 3	Forward 5'-GACTGGGTGTGGGGAGGT-3' Reverse 5'-GGACCGAGAGGAGTCTCAAA-3'	59°C for 30 sec
exon 4, 5	Forward 5'-CTTCTCCCTCTGCCTGTGTC-3' Reverse 5'-GGGAGGTTGAAGGAGGAGAA-3'	58°C for 60 sec
exon 6	Forward 5'-GGCTTTCTCCTCCTTCAACC-3' Reverse 5'-ATTCCACATTGGGACAGGAA-3'	58°C for 30 sec
exon 7, 8	Forward 5'-TCTGTAGGCTTTGGCTCTACG-3' Reverse 5'-CTAGCCTCACATGTGCTCCA-3'	60°C for 60 sec
exon 9	Forward 5'-TGCTGCAGATTGGCACTTAC-3' Reverse 5'-GGATCAGTTCCCTTCCACCT-3'	59°C for 30 sec
exon 10	Forward 5'-GGACCCTTCCAAGCCTAGAT-3' Reverse 5'-ACACCGGAATAGGTGTGCTC-3'	59°C for 30 sec

Isolation of DNA from peripheral blood mononuclear cells and its sequencing. Genomic DNA was extracted from the whole peripheral blood using QIAamp DNA Blood Mini Kit from Qiagen, according to the manufacturer's instructions. The DNA was re-suspended in 50 µl of Qiagen elution buffer AE and stored at –20°C. *TP53* gene polymorphisms were determined by polymerase chain reaction (PCR) amplification with primers designed using Primer3 software. Exons 2 to 10 of *TP53* gene were amplified including about 60-bp flanking regions of each exon. We did not manage to amplify exon 1 due to technical difficulties. The primers' sequences and PCR conditions are shown in Table 1. The reactions were carried out in a volume of 12.5 µl containing: 10 × Taq DNA Polymerase buffer with $MgCl_2$, 5 × GC-rich solution, 0.24 mM dNTPs, 0.5 µM of the primers, 1 unit of Taq Polymerase (Roche) and 40-60 ng of genomic DNA. The PCR cycle conditions were as follows: an initial denaturation at 95°C for 30 sec, annealing at temperature shown in Table 1 for 1 min and elongation at 72°C for 30/60 sec, with a final extension at 72°C for 7 min. PCR products were separated by electrophoresis in 1.5% agarose gel, purified on Millipore plates and used as templates for a sequencing reaction, based on Sanger's method (17). The re-amplification was performed using one specific primer (in forward direction for exons 2, 3, 6, 9 and 10, and in both forward and reverse directions for exons 4, 5, 7 and 8). Re-amplification products were purified using EDTA and ethanol precipitation and separated by capillary electrophoresis using ABI 3130 sequencer from Applied Biosystems.

Statistical analysis. On the basis of the obtained sequences of the analyzed gene regions, single nucleotide changes were assessed and calculation of the Chi-square test for deviation from Hardy-Weinberg equilibrium (HWE) was performed. Genotype and allele frequencies were obtained and Fisher's exact test was used for comparison. Additionally, odds ratio values (OR) were evaluated with 95% confidence intervals (95% CI). *TP53* gene structure was generated using commercially available Haploview 3.2 software (http://www.broad.mit.edu/mpg/haploview/) and the pairwise linkage disequilibrium (LD) was calculated as R^2 value.

Results and discussion

Based on sequencing analysis, the genotypes of *TP53* gene were determined. There were two new single nucleotide changes in the coding region of exon 3 and intron 7, respectively. One single nucleotide change, named c.213C>T, occurred in the control group and was located at the 213 position in mRNA, where position 1 refers to the first base in ATG start codon, and did not result in an amino acid change (p.Ser103Ser). The other, ex7+150T>C, occurred both in control and tumor groups and it was located 150 bp downstream of the last nucleotide of exon 7. Both variants were heterozygous and frequencies of alleles and genotypes in both positions were determined. Distribution of *TP53* genotypes was consistent with HWE (data not shown). The frequency of alleles and genotypes of *TP53* gene variants in tumor patient and control groups are summarized in Table 2. The odds ratio values, calculated for the less frequent allele and genotype in each group, revealed no significant differences in the allelic and genotypic frequencies either between control groups or between control and investigated tumor groups. Despite the lack of statistical significance, there was a higher prevalence of allele T in the c.213C>T change in controls compared to tumor affected bitches (OR = 1.9, p = 1.0). However, the incidence of the allele T was more pronounced in comparison to the age-groups of controls (OR = 3.0, p = 1.0 for geriatric

Tab. 2. Distribution of *TP53* genotype and allele frequencies

TP53		Tumor Patients	Controls		p value	OR (95% CI)
c.213C>T	Genotype frequency	(n = 22)	(n = 36)			
	CC	22 (1.0)	35 (0.97)			
	CT	0 (0)	1 (0.03)		1.0	1.9 (0.074-48.78)
			group G[1] (n = 11)			
			CC 10 (0.91)			
			CT 1 (0.09)	G vs. A	1.0	3.0 (0.109-82.403)
			group A[2] (n = 10)			
			CC 10 (1.0)			
			CT 0 (0)	A vs. Y	1.0	1.48 (0.027-80.47)
			group Y[3] (n = 15)			
			CC 15 (1.0)			
			CT 0 (0)	G vs. Y	0.4231	4.43 (0.164-119.49)
	Allele frequency					
	C	44 (1.0)	71 (0.99)			
	T	0 (0)	1 (0.01)		1.0	1.72 (0.069-43.22)
			group G			
			C 21 (0.95)			
			T 1 (0.05)	G vs. A	1.0	2.86 (0.11-74.37)
			group A			
			C 20 (1.0)			
			T 0 (0)	A vs. Y	1.0	1.49 (0.028-78.1)
			group Y			
			C 30 (1.0)			
			T 0 (0)	G vs. Y	0.4231	4.26 (0.165-109.6)
ex7+150T>C	Genotype frequency	(n = 22)	(n = 36)			
	TT	20 (0.91)	27 (0.75)			
	CT	2 (0.09)	9 (0.25)		0.1783	3.33 (0.65-17.15)
			group G (n = 11)			
			TT 8 (0.73)			
			CT 3 (0.27)	G vs. A	1.0	0.88 (0.13-5.82)
			group A (n = 10)			
			TT 7 (0.7)			
			CT 3 (0.3)	A vs. Y	0.6532	1.7 (0.27-10.93)
			group Y (n = 15)			
			TT 12 (0.8)			
			CT 3 (0.2)	G vs. Y	1.0	1.5 (0.24-9.39)
	Allele frequency					
	T	42 (0.95)	63 (0.875)			
	C	2 (0.05)	9 (0.125)		0.2024	3.0 (0.62-14.59)
			group G			
			T 19 (0.86)			
			C 3 (0.14)	G vs. A	1.0	0.89 (0.16-5.04)
			group A			
			T 17 (0.85)			
			C 3 (0.15)	A vs. Y	0.6723	1.59 (0.29-8.8)
			group Y			
			T 27 (0.9)			
			C 3 (0.1)	G vs. Y	0.6890	1.42 (0.26-7.82)

Explanations: [1] geriatric group, [2] adult group, [3] young group

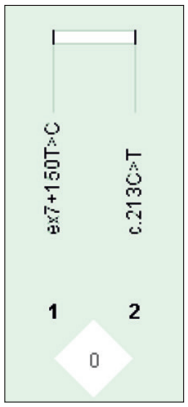

Fig. 2. Linkage disequilibrium plot for *TP53* gene in controls
Explanation: *TP53* gene structure was generated using commercially available Haploview 3.2 software (http://www.broad.mit.edu/mpg/haploview/) and the pairwise linkage disequilibrium (LD) was calculated as R^2 value.

vs. adult, and OR = 4.43, p = 0.4231 for geriatric *vs*. young). This indicates an increased incidence of the T allele in bitches of lower age. Furthermore, no statistical significances were observed for differences in genotypic and allelic frequencies linked to ex7+150T>C changes comparing control and tumor patient groups, and control age-groups. Nevertheless, the prevalence of allele C (OR = 3.0, p = 0.2024) and those of genotype CT (OR = 3.33, p = 0.1783) were 3-fold higher in controls compared to the tumor group.

The construction of gene structures failed in tumor bitches due to the undifferentiated genotypes within the c.213C>T change. *TP53* gene structure analysis in the control group revealed a complete lack of linkage between c.213C>T and ex7+150T>C changes ($R^2 = 0$). The LD plot for analyzed single nucleotide changes is shown in Figure 2.

Tumor growth, invasion, progression as well as the entire process of carcinogenesis is regulated by a disturbed cluster of target gene expression which leads to changes in cell cycle division and cell metabolism (5, 9). Moreover, tumor development is also dependent on aberrations in a DNA sequence and/or improper DNA repair mechanisms in the cell. Recently, several data have been published, indicating the incidence of new mutations and the association of specific polymorphisms with human cancers (3, 12, 22). Although, the available DNA sequencing methods permit the detection of mutations, there are still only few reports indicating links with canine tumor incidence. The *TP53* gene encodes a protein responsible for the transcription of genes which regulates among others cell cycle and cell division. As demonstrated in human cancers, mutation in the gene encoding TP53 protein significantly influenced the manifestation of uncontrolled cell divisions and induction of tumor growth. It was also stated that malfunction in *TP53* gene expression or incidence of polymorphisms may be correlated with cell senescence and aging (4, 11, 15, 20). In our study, we have analyzed mutations in the *TP53* gene in bitches with diagnosed mammary adenocarcinomas and in healthy females of different ages. We identified two polymorphic variants in exon 3 and in intron 7 of the *TP53* gene. We did not find differences in allele or genotype distribution between the tumor and the control groups. However, we suppose that the occurrence of the C allele may play a protective role with regard to the incidence of mammary carcinoma in bitches.

In this study we found single nucleotide polymorphism in the non-coding sequence of intron 7. This change does not significantly influence the amino acid sequence and protein synthesis. However, it is a well documented theory that changes in the non-coding sequence of introns may significantly affect mRNA expression, especially in the case of several transcription factors binding sites. Therefore, we may suggest that the polymorphism in intron 7 observed in our study may lead to disregulation in TP53 transcript expression, which may be a main reason of dysfunction in cell cycle division as well as lead to cancerogenesis induction.

The occurrence of *TP53* polymorphisms in canines was studied by Rivera et al. (14). Ten candidate genes which may be related to human mammary gland tumor development were investigated and the results were compared with the appearance of CMT in English Springer Spaniels. No association was observed between *TP53* polymorphisms and the incidence of tumor in 212 CMT and 143 control dogs. However, a significant association was found between *BRCA1* and *BRCA2* gene polymorphisms and CMT. It was concluded that this type of tumor may be a good model for human breast cancer. In a further study, Borge et al. (1) investigated the association between CMT and incidence of polymorphisms in eleven candidate genes (*BRCA1*, *BRCA2*, *BRIP1*, *CDH1*, *CHEK2*, *EGFR*, *ESR1*, *HER2*, *PTEN*, *STK11* and *TP53*) to determine the genetic risk factors for CMT development in bitches. They found 46 single nucleotide polymorphisms (SNPs); nine of the coding SNPs were non-synonymous, where four of them were located in gene regions conserved across the four breeds. It was concluded that the genetic changes differed significantly in the studied breeds and, therefore, it was difficult to clearly define the genetic risk of CMT in canines.

Although *TP53* gene polymorphisms were identified in several canine tumors, such as transmissible venereal tumor, osteosarcoma or brain tumors, there are only two reports which partially recognize an association between *TP53* gene polymorphisms and the incidence of canine mammary gland adenocarcinoma or aging, respectively (1, 14). Since it was clearly demonstrated in humans that *TP53* gene polymorphic variants are linked with several tumor types, it seems to be rational to investigate similar associations with canine mammary adenocarcinoma. Our results may confirm that the occurrence of allele C could represent a protective factor against the incidence of mammary gland adenocarcinoma and the *TP53* polymorphisms are linked to mammary tumors in canines. Since we did not observe an association between c.213C>T and ex7+150T>C polymorphisms of the *TP53* gene in the control group, it may be suggested that these polymorphisms are not related to canine aging.

References

1. *Borge K. S., Børresen-Dale A. L., Lingaas F.*: Identification of genetic variation in 11 candidate genes of canine mammary tumour. Vet. Comp. Oncol. 2011, 9, 241-250.
2. *Bourdon J. C.*: p53 and its isoforms in cancer. Br. J. Cancer 2007, 97, 277-282.
3. *Chen X., Jorgenson E., Cheung S. T.*: New tools for functional genomic analysis. Drug Discov. Today 2009, 14, 754-760.
4. *Diderich K., Alanazi M., Hoeijmakers J. H.*: Premature aging and cancer in nucleotide excision repair-disorders. DNA Repair (Amst) 2011, 10, 772-780.
5. *Im K. S., Kim I. H., Kim N. H., Lim H. Y., Kim J. H., Sur J. H.*: Breed-related differences in altered BRCA1 expression, phenotype and subtype in malignant canine mammary tumours. Vet. 2012, 195, 366-372.
6. *Jugdutt B. I., Jelani A., Palaniyappan A., Idikio H., Uweira R. E., Menon V., Jugdutt C. E.*: Aging-related early changes in markers of ventricular and matrix remodeling after reperfused ST-segment elevation myocardial infarction in the canine model: effect of early therapy with an angiotensin II type 1 receptor blocker. Circulation 2010, 122, 341-351.
7. *Kirpensteijn J., Kik M., Teske E., Rutteman G. R.*: TP53 gene mutations in canine osteosarcoma. Vet. Surg. 2008, 37, 454-460.
8. *Klopfleisch R., von Euler H., Sarli G., Pinho S. S., Gärtner F., Gruber A. D.*: Molecular carcinogenesis of canine mammary tumours: news from an old disease. Vet. Pathol. 2011, 48, 98-116.
9. *Król M., Pawłowski K. M., Majchrzak K., Gajewska M., Majewska A., Motyl T.*: Global gene expression profiles of canine macrophages and canine mammary cancer cells grown as a co-culture in vitro. BMC Vet. Res. 2012, 8, 16.
10. *Langdon S. P.*: Animal modeling of cancer pathology and studying tumour response to therapy. Curr. Drug Targets 2012, 13, 1535-1547.
11. *Leroy B., Fournier J. L., Ishioka C., Monti P., Inga A., Fronza G., Soussi T.*: The TP53 website: an integrative resource centre for the TP53 mutation database and TP53 mutant analysis. Nucleic Acids Res. 2013, 41, D962-D969.
12. *Lindgren D., Sjödahl G., Lauss M., Staaf J., Chebil G., Lövgreen K., Gudjonsson S., Liedberg F., Patschan O., Månsson W., Fernö M., Höglund M.*: Integrated genomic and gene expression profiling identifies two major genomic circuits in urothelial carcinoma. PLoS One 2012, 7, e38863.
13. *Misdorp W., Else R. W., Hellmén E., Lipscomb T. P.*: Histological classification of mammary tumors of the dog and the cat. Published by the Armed Forces Institute of Pathology and the American registry of Pathology and The World Health Organization Collaborating Centre for Worldwide Reference on Comparative Oncology, Washington, D.C., USA 1999, 7, 11-29.
14. *Rivera P., Melin M., Biagi T., Fall T., Häggström J., Lindblad-Toh K., von Euler H.*: Mammary tumour development in dogs is associated with BRCA1 and BRCA2. Cancer Res. 2009, 69, 8770-8774.
15. *Rivera P., von Euler H.*: Molecular aspects on canine and human mammary tumours. Vet. Pathol. 2011, 48, 132-146.
16. *Sánchez-Servín A., Martínez S., Córdova-Alarcon E., Fajardo R.*: TP53 polymorphisms allow for genetic sub-grouping of the canine transmissible venereal tumour. J. Vet. Sci. 2009, 10, 353-355.
17. *Sanger F., Nicklen S., Coulson A. R.*: DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 1977, 74, 5463-5467.
18. *Stockmann D., Ferrari H. F., Andrade A. L., Cardoso T. C., Luvizotto M. C.*: Detection of the tumour suppressor gene TP53 and expression of p53, Bcl-2 and p63 proteins in canine transmissible venereal tumour. Vet. Comp. Oncol. 2011, 9, 251-259.
19. *Toledo F., Wahl G. M.*: Regulating the p53 pathway: in vitro hypotheses, in vitro veritas. Nat. Rev. Cancer 2006, 6, 909-923.
20. *Tornesello M. L., Buonaguro L., Buonaguro F. M.*: Mutations of the TP53 gene in adenocarcinoma and squamous cell carcinoma of the cervix: A systematic review. Gynecol. Oncol. 2012, 128, 442-448.
21. *Vousden K. H., Lane D. P.*: p53 in health and disease. Nat. Rev. Mol. Cell Biol. 2007, 8, 275-283.
22. *Waters M. D., Jackson M., Lea I.*: Characterizing and predicting carcinogenicity and mode of action using conventional toxicogenomics methods. Mutat. Res. 2010, 705, 184-200.
23. *Wells D.*: Dogs as a diagnostic tool for ill health in humans. Altern. Ther. Health Med. 2012, 18, 12-17.
24. *York D., Higgins R. J., LeCouteur R. A., Wolfe A. N., Grahn R., Olby N., Campbell M., Dickinson P. J.*: TP53 mutations in canine brain tumors. Vet. Pathol. 2012, 49, 796-801.

Corresponding author: Bartosz Kempisty PhD, ul. Święcickiego 6, 60-781 Poznań, Poland; e-mail: etok@op.pl