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MOLECULAR BIOLOGY OF THE ADENOMATOUS POLYPOSIS COLI GENE: IMPLICATIONS FOR GENETIC TESTING

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Abstract

Germ-line transmitted mutations of the adenomatous polyposis coli (APC) tumor suppressor gene predispose gene carriers to development of multiple colorectal adenomas, so called familial adenomatous polyposis (FAP), which are prone to malignant progression. In this article, we will show the role of tumor suppressor genes for the development of hereditary cancer syndrome and will focus on the FAP and APC. We refer about the methods and results of genetic testing. Genetic alterations are spread over the whole APC gene and therefore testing of the complete gene is often required to identify the inactivating mutation in APC index patient. The vast majority of genetic alterations inactivating the APC gene lead to premature stop of translation. As a consequence, binding domains of the APC protein are lost in most colorectal tumors. Evidence is accumulating that the APC protein is intimately involved in physiological processes related to migration, proliferation, adhesion and differentiation. These multiple functions are realized by an apparently significant functional complexity of the APC gene and its products. Alternative splicing represents an important mechanism contributing to structural heterogeneity of APC transcripts, as we show in our results.

Key words: APC, FAP, genetic testing, alternative splicing

1. INTRODUCTION

Our understanding of the biology of cancer increased dramatically. It is now widely recognized that cancer is caused by deregulation of growth controlling molecular signal transduction pathways, due to mutations in genes coding for the protein components of these pathways. The mutation results in inactivation or aberrant expression of these genes (1). That cancer in man might be inherited has been assumed for over a century. Hereditary cancers are caused by inheritance of germ line mutations and account for 5-10 % of all cancers. Cardinal feature of hereditary cancer syndromes include early age of cancer onset, which is frequently bilateral, or multifocal, multiple primary cancers, segregation of the same cancer type within the pedigree, vertical transmission through either parent (2). Recent advances in genetic testing and molecular diagnosis have contributed to accurate phenotype-genotype correlation, which is essential for characterization of hereditary cancer syndromes and for targeted surveillance and management strategies in accordance with the natural history of these hereditary cancers.

2. TUMOR SUPPRESSOR GENES: THE BRAKES OF TUMOR DEVELOPMENT AND CLUE TO HEREDITARY CANCER SYNDROMES

Evidence for the existence of genes that could suppress tumor development came from different directions. As early as 1969, Harris and Goss showed that tumor cells lost their malignant characteristics when they were fused to normal fibroblasts (3). This suggested that genes of normal cells were able to suppress malignancy. Whereas protooncogenes are activated by a mutation, tumor suppressor genes are defined by their inactivation in human cancer (4). Enormous progress has been made in the identification of inherited and somatic mutations in tumor suppressor genes in human cancer, as well as in defining the means by which inactivating (loss-of-function) mutations in these genes contribute to the development of cancer.

The epidemiologist Alfred Knudson initiated the studies leading to the identification of the first tumor suppressor gene (5). He noticed that hereditary cases of retinoblastoma, a malignant

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childhood tumor of the retina, appeared at an earlier age and were more often bilateral and multifocal than in sporadic (= non-hereditary) cases. Based on this observation, he formulated his now famous „two hit hypothesis“ which suggested that two inactivating mutational events were necessary for compromising tumor suppressor function. In hereditary cases, the first mutation („first hit“) provides the genetic susceptibility that is transmitted via the sex cell to the next generation (germ line mutation). Consequently this mutation is present in all somatic cells, including the retina cells, of the carrier.

The early age of onset of the hereditary form of the disease is compatible with the requirement for one additional mutational event („second hit“) to initiate tumorigenesis. The consequence is that the disease is autosomal dominantly inherited but the cancer phenotype is a result of recessive action of the mutation (loss-of-function) (5,8). In sporadic cases both mutations would occur within the normal retina cells and the lower probability of this explains the later age of onset and the unilaterality of the disease.

An important refinement of the Knudson model was the notion that the mutations could target both alleles of the same gene. The retinoblastoma was the first tumor suppressor gene to be successfully isolated (6). This was a milestone in cancer genetics. The Rb1 gene codes for a protein that serves as a kind of handbrake in the cell division cycle. Cells can only enter or progress through the cell cycle when the retinoblastoma protein is temporarily inactivated. In normal cells this is a tightly controlled process, but mutations in the Rb1 gene may result in loss of the brake function, permitting uncontrolled growth (7).

Thus far, about 30 tumor suppressor genes have been identified and definitely implicated in cancer development. Germline-inactivating mutations in some of these genes are associated with inherited cancer predisposition, inclusive hereditary colorectal cancer (8).

3. HEREDITARY COLORECTAL CANCER SYNDROMES

Hereditary colorectal cancer syndromes are usually subdivided into polyposis and nonpolyposis types (2). The polyposis types are those in which dozen or even thousands of benign tumors (polyps) are often seen prior to cancer development. In the nonpolyposis types, few if any polyps are noted prior to cancer formation and this type is called hereditary nonpolyposis colorectal cancer (HNPCC). For this type of syndromes, the microsatellite instability is a typical trait. The microsatellite instability is characterized by the size variation of microsatellite DNA in tumor DNA as compared to matching normal mucosa due to defects in the mismatch repair system (MMR). The molecular diagnosis of HNPCC is based on determining of MMR genes for germ line mutations (2). There are at least six different proteins required for the complete MMR system (hMSH2, hMLH1, hPMS1, hPMS2, hMSH3 and hMSH6).

There are more polyposis syndromes. One of the polyposis syndromes is known as familial adenomatous polyposis (FAP) or adenomatous polyposis coli (APC) (2, 10).

FAP is an autosomal dominant inherited disorder affecting about 1 in 8000 individuals. The syndrome is characterized by the development of hundreds of adenomatous polyps in the colon and rectum of affected individuals by early adulthood. The lifetime risk of colorectal cancer in those with the classic form of FAP is extremely high, approaching nearly 100% by the age 60. The gene APC, responsible for FAP, was mapped to chromosome 5 and cloned and sequenced by independent groups (9).

There are some other tumor suppressor genes that were identified to be involved in different hereditary colorectal cancer syndromes. Peutz-Jeghers syndrome is an autosomal dominant inherited disease with a genetic predisposition caused by a mutation within the gene LKB1/STK11, Cowden disease is associated with a mutation in the PTEN gene, familial juvenile polyposis syndrome is genetically predisposed by mutations in DPC4, PTEN and other genes (10).

4. FAP AND THE APC GENE

The first clue to localizing the position of the APC gene came from identification of a patient with colorectal polyposis and mental retardation that had a deletion of the chromosomal band

5q21 (11). Linkage analysis of families with FAP led to the mapping of the gene to 5q21 and its cloning (12,13). The APC gene consists of 8535 base pairs in 21 exons that encode a protein with the size of 2843 amino acids. The largest exon 15 comprises more than 75% of the coding sequence and is the target of most germ line mutations in FAP patients. The vast majority are nonsense or frame shift mutations that result in a truncated protein product (14). The consequence is loss of function and loss of tumor suppressor activity. As expected from Knudsen's „two hit hypothesis“, colorectal tumors from FAP patients nearly all harbor either additional somatic APC mutations („second hit“) or loss of the heterozygosity at the APC locus („second hit“), in addition to the original germ line mutations („first hit“) (15). More than 1400 different disease-associated mutations of the APC gene have been reported. Most of these are insertions, deletions, and nonsense mutations that lead to frame shift and/or premature stop codons. Most mutations that cause classic FAP occur in APC between codons 169 and 1393, with the most common APC mutations in codon 1309 and 1061, respectively. These so called „mutation hot spots“ lie within the mutations cluster region, the region with the most mutations between the codons 1286 and 1513 (16).

5. METHODS FOR DETECTING THE GERM LINE MUTATIONS WITHIN THE APC GENE

The worldwide mutational screening for the APC mutations leads to the development of several very effective DNA - based test for APC germ line mutations including denaturing gradient gel electrophoresis (DGGE), heteroduplex analysis (HDA), single strand conformation polymorphism (SSCP), protein truncation test (PTT) and DNA sequencing. Among these various techniques developed for mutation detection the SSCP and HDA are widely used. SSCP detects base changes in single-stranded DNA, whereas HDA does the same in double-stranded DNA subjected to electrophoresis in non-denaturing conditions (17).

The basic method for all these methods is the polymerase chain reaction (PCR). The exon 15 has to be divided into the 300-400 base pairs pieces, the corresponding primers were created and the appropriate DNA was amplified by the PCR. Because the coding exons 1 to 14 of the APC gene have an appropriate size for a PCR product, the whole exons including splice regions were amplified by PCR. In SSCP analysis, the PCR product is denatured, and separated strands adopt folded structures determined by their nucleotide sequences. A single base alteration is detected by SSCP when the folding of the single strand changes is sufficient to alter its electrophoretic mobility. In HDA, the PCR-amplified DNA fragments are denatured and re-annealed to give a mixture of four duplexes, two homoduplexes and two heteroduplexes in the heterozygote samples (18). The final identification of the mutation have to be performed by direct DNA sequencing.

Some laboratories use protein truncation test (PTT), because most APC mutations cause premature truncation of the APC protein (19,20). The genomic DNA extracted from blood is used to perform this assay and exon 15 is amplified in segments about 1000 bp using PCR primers with T7 promoter sequence that is used to initiate transcription by the T7 RNA polymerase. The in vitro transcription/translation are performed within the same tube and the ³⁵S-labeled methionine is used to label the in vitro protein products. For analyzing the exons 1-14 by PTT, isolation of RNA and RT-PCR is necessary. The in vitro transcription /translation is performed as mentioned above. The labeled protein products are then analyzed by polyacrylamide gel electrophoresis. The in vitro protein product from a healthy person will run at the expected size. The protein product from an index patient with FAP that has a nonsense mutation within this gene segment will run at a smaller size because it is truncated. After PTT, the confirmation of the mutation by direct DNA sequencing is also necessary.

6. FAP AND PHENOTYPE-GENOTYPE CORRELATION

In FAP, a clear correlation between specific clinical features and the locations of the mutation in the APC gene, termed genotype-correlation, has been observed. APC germ line mutations

between codons 463 and 1387 are associated with congenital hypertrophy of the pigmented retinal epithelium, and mutations between 1445 and 1578 are associated with an increased risk of desmoid tumors (9). In addition, mutations at the 5' end of the gene proximal to the 1517 or at the 3' end of the gene distal to codon 1900 are associated with attenuated FAP (9). These features are not uniform, and in fact, it is likely that modifier genes, which are unidentified at this time, also play a significant role in affecting the severity of the FAP. Nonetheless, these genotype-phenotype correlations have the potential to increase the effectiveness of screening, surveillance, and treatment by identifying people who should be considered for modified surveillance and treatment.

7. THE ROLE OF APC IN TUMORIGENESIS: STRUCTURAL ANALYSIS OF THE APC PROTEIN AND PROTEIN PARTNERS

The APC gene product, known as APC protein, is large and has a size of 2.843 amino acids and a molecular mass of 310 kDa (21). Functional analysis has revealed a broad spectrum of interaction with several protein and cellular components. Our current understanding of functions of the APC protein comes from analyzing of putative functional motifs and from detection of its protein partners. The first exon of the APC gene encodes a protein domain capable to create homodimer with another APC protein molecule *in vitro* (22). Homodimerization of the APC at the amino-terminus implies a possible dominant-negative mode of action for mutant APC in heterozygous cells, in which shorter (mutant) proteins can functionally inactivate the normal full-length (wild type) protein (23). This domain is followed by armadillo repeat region which interacts with several proteins – APC-stimulated exchange factor (ASEF), KAP3A which belongs to the kinesin superfamily and with the regulatory subunit of the phosphatase 2A (24). The central region of the APC contains three 15-amino-acids repeats and seven 20-amino acid repeats which all bind to β -catenin. The 20 amino acid repeats are essential for downregulation of β -catenin. The three so-called SAMP (serine, alanine, methionine proline) repeats lie between the β -catenin binding domains and bind to the tumor suppressor proteins conductin or axin. In most colon tumors SAMP are deleted because they are located C-terminal to the MCR and the truncated APC protein is not able to bind axin and downregulate β -catenin (24). Five nuclear export signals (NES) were found in APC. APC can shuttle between the cytoplasm and the nucleus, which could be one of the roles of the NES (25). Two domains of the APC at the carboxy terminus are involved in the association with microtubules; the basic region probably interacts directly with microtubules, whereas a more carboxy-terminal end binds to EB1, a microtubule-binding protein (26). The best-characterized function of APC is its role as a negative regulator of the Wnt signaling pathway via its interaction with β -catenin (23,24). β -catenin is degraded after phosphorylation. This is only possible in a multiprotein complex consisting APC, GSK3b, β -catenin and conductin/axin. The free β -catenin is accumulated in the cytoplasm and its degradation blocked when this complex has not been established due to the mutation in the APC gene. As a consequence, the β -catenin is then translocated to the nucleus and bound to the T-cell factors/lymphoid enhancer factors (TCFs/LEFs) that activate the transcription of target genes involved in differentiation and proliferation (23). The consequence is an uncontrolled cell growth. The evidence is accumulating that the APC protein is intimately involved in physiological processes related to migration, proliferation, apoptosis and differentiation (24). These multiple functions are realized by an apparently significant structural and functional complexity of the APC gene and its products.

8. APC AND ALTERNATIVE SPLICING

In the 1977, it was detected that eucaryotic genes consist of intervening sequences, which have to be removed after transcription (27). These intervening sequences were named introns and represent non-coding regions of the DNA. The DNA gene segments, which were separated by introns and represent coding sequences creating mature mRNA, were named exons. The process

of excision of introns and connection of exons was named splicing. The constitutive splicing represents the connection of exons according to their linear sequence. Conversely, during alternative splicing some exons may be skipped in the mature mRNA (28).

Alternative splicing represents an important mechanism contributing to structural heterogeneity of APC transcripts. In our studies we focused on alternative splicing affecting the coding exons 8 to 15 of the APC gene. The first evidence for alternative splicing as a mechanism for generation of diversity was the detection of alternatively spliced exon 9, exon 7 and the 5'UTRs (12, 29, 30). Our experiments detected new exon connections contributing to the mRNA heterogeneity – an in frame insertion of a 54 nucleotide sequence encoded by a novel exon located 1.6 kb down-stream from exon 10 named 10A and skipping of exon 14, resulting in a novel exon 13 to 15 connection (31). We characterized exon 10A – it is highly conserved, alternatively spliced, inserted into mature transcripts and expressed in a tissue-independent fashion. We presented exon 10A flanking sequences so that this novel exon can be included in future mutations screening procedures (32). The exon 14 skipping provided the mRNA with a novel open reading frame, which was terminated after 19 codons of exon 15-derived protein sequence. A panel of 8 different transcripts specified by their unique exon composition has been distinguished by cloning a sequencing analysis. Combinatorial joining of exon 9, the novel exon 10A and exon 14 generated this complexity (31). RT-PCR expression analyses were carried out to demonstrate that this complexity of splice variants is synthesized in cell lines from various tissues and independent of malignancy. Taken together, we have provided evidence that additional exon sequences and novel splice mechanism contribute to the structural and possibly the functional complexity of the APC gene (31). To prove that exon 14-skipped mRNA variants do not simply represent tissue culture artifacts, expression of these APC transcripts was demonstrated in native colorectal epithelium (33). We concluded that APC exon 14 skipped transcripts are physiologically expressed in native human colon mucosa and that ratios of exon 14-negative to exon 14-positive isoforms were not altered when colorectal tumor cells were compared with matching normal mucosa.

One of the numerous splice events affects exon 14 of the APC gene as mentioned above. The important biological significance of exon 14 skipping was highlighted by a FAP kindred with early onset of disease due to constitutive overexpression of the APC exon 14-deficient molecules as the result of a germinal exon 14 splice acceptor defect (34). APC exon 14-negative transcripts code for low molecular weight APC proteins of approximately 67 kDa with a short C-terminal rail of 19 amino acids instead of the exon 15 encoded long open reading frame. Intracellular stable low molecular weight APC proteins of expected size have been identified immunochemically in Epstein Barr virus (EBV)-immortalized B-cells of these FAP patients, which could be the clue to the physiological role of these protein isoforms.

The exons at the 5'end of the APC gene were called UTR's. Recent studies showed that exon 1 could be skipped and exon BS, preliminarily known as untranslated region, is translated into a functional APC protein without exon 1 in differentiated neuronal tissue (35). We identified 4 novel APC mRNA isoforms, which are characterized by novel 1A/BS connections in combination with exon BS/2 junction, giving rise to alternative amino terminal open reading frames without exon 1. These APC exon BS/2 connection-specific transcripts predominantly exhibit concomitant expression of exons 9 and 10A, suggesting the gene product with an extended heptad repeat (36).

9. CONCLUSION

In our studies, we gave evidence of complex alternative splicing of the pre-mRNA of the APC gene. The mRNA isoforms with exon 1 were combined with all alternative spliced exons, including the "light chains" of the APC protein. Conversely, the exon 1-skipping isoforms exhibit concomitant expression of exons 9, 10A and 14. Others and we detected a stable existence of alternative protein isoforms without exon 1 and without exon 14, respectively. We concluded from the evidence that these proteins are functionally active. The germ-line mutations of exon 14 splice acceptor site is suggested to be involved in a severe phenotype of FAP, the physiological function

of these isoforms in epithelial cell remains unknown. It is possible that they act as negative regulators of the wild-type APC during certain parts of the cell cycle.

The identification of the APC gene has been translated into clinical practice and has led to improvement of risk assessment through the use of genetic testing. The introduction of genetic testing for the assessment of FAP risk has led to more effective management strategies for patients with potentially high-risk colon cancer in Slovakia too (37). As discussed above, the APC gene encodes for a multifunctional protein. Many of other tumor suppressor genes have similarly been shown to encode for multifunctional proteins that possibly coordinate signal transduction with other cellular activities. In the gut, the APC protein controls cellular proliferation, adhesion, migration and differentiation in the self-renewing crypts and villi. This important regulatory role is reflected by the consequences of APC mutation in intestinal tumor initiation and progression. There is no doubt concerning the functionality of the protein products of alternative splicing, but the importance of alternative splicing for the protein multi-functionality remains to be determined.

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MORPHOLOGY IN FULL-TERM PHYSIOLOGICAL NEONATES OF ROMANIES (GYPSIES) FROM WESTERN SLOVAKIA

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Abstract

Values of body dimensions permit the evaluation of body growth, as the principal indicator of health and socio-economic status of individuals and population groups. Research of body dimensions in neonates and children in precisely defined ethnic groups is important because permits follow-up variations in growth and development caused by genetic and environmental factors. Romanies (Gypsies) are the second largest nationality group in Slovakia.

We have anthropologically examined 95 full-term physiological Romany neonates. Control group of non-Romany neonates consisted of 221 subjects. Statistically we have evaluated 16 somatometric indicators and 5 body indexes.

Romany neonates in both genders have significantly lower birth weight and shorter body length as non-Romany neonates. Significantly lower values in Romany neonates were noted also in head and chest circumference, width of the cranial basis, biacromial width, sagittal diameter of thorax and also in circumference of the thigh. In Romany girls we have noted significantly lower abdominal, arm and calf circumference, maximum length of head, and thoracic index. Generally, Romany neonates have significantly higher cephalic index – they are brachycephalic, while non-Romanies are mesocephalic.

Key words: Romany and non-Romany neonates, anthropometrical dimensions, body measurement indexes, comparison

INTRODUCTION

Values of body dimensions permit the evaluation of body growth as the main indicator of health and socio-economic status of individuals and consequently of population groups (1). The values of examined somatometric indicators are of low-information value when not compared with standard or reference data. Anthropological research of body dimensions in neonates and children in precisely defined ethnic groups is under serious attentions and considered as important, because it permits to follow up variations in growth and development caused by genetic and environmental factors (2).

From ethnical point of view, Romanies are submitted to Europoid population. Their original home is located to Central Northern India. With their body characteristics they do not form and integral complex, it is possible to differentiate various types. The prevalence of dark pigmentation of hair and eyes and tawny pigmentation of skin is characteristic (3). Romanies are the second numerous ethnical minority in Slovakia. In the addition, in May 2001 89,920 Slovak citizens have enrolled their membership to the Romany nationality, which is 1.7 % of Slovak population, but the real number of Romanies is estimated to be many times higher. According to the data of local authorities from 1989, there were 253,943 Romanies in Slovakia. These statistics registered only socially dependent citizens, so it is expected that real number of Romanies in Slo-

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vakia is markedly higher. Today the estimations of experts present the number of 480,000-520,000, however, with regard to the very high natality of Romanies this number is still increasing (4).

The problems of Romany citizens have very high social impact and are manifested not only in the social sphere, but also to the sphere of healthcare. Alarming is the situation of health status in the Romany settlements. In the past period there was notable increase in diseases of respiratory system, dermatological diseases and especially sexually transmitted diseases. In children, infectious and parasitic diseases are frequent, that are not present in the majority population (4). The main causality of a bad health status consists in long-term bad economical situation, low educational level and incorrect lifestyle of the Romany minority. Total premature mortality in the Romanies are probably three times higher than in the Slovak population (5).

METHODS

Somatometric measurements were taken at the Department of Neonatology, Slovak Medical University and Hospital in Nové Zámky, Slovakia (Head of Dept.: Ass. Prof. František Bauer, M.D., Ph.D.), Department of Neonatology, Hospital in Komárno, Slovakia (Chief Physician: Ingrid Moravcová, M.D.) and Dept. of Neonatology, Faculty Hospital of L. Dérer in Bratislava, Slovakia (Head Of Dept.: Ass. Prof. Anna Kardošová, M.D., PhD. replaced by Ass. Prof. Helena Drobná, M.D., PhD.) in 2001 - 2005. During this period, we have examined and statistically evaluated 95 full-term physiological neonates of the Romanies (53 boys and 42 girls). The control group consisted of 221 full-term physiological non-Romany neonates (122 boys and 99 girls).

Table 1 : Gestation age of examined neonates

Gestation age (weeks)	Boys			Girls		
	N	Mean	SD	N	Mean	SD
Romany newborns	53	39.55	1.00	42	39.45	0.91
Non-Romany newborns	122	39.74	1.08	99	39.94	0.96

Neonates born between the 38th till 42nd week of gestation. The average gestation age in the group of Romany neonates was 39.55 weeks in boys and 39.45 weeks in girls. In non-Romany neonates, the gestation age in boys was 39.74 weeks and 39.94 weeks in girls. Gestation age was determined by the gynecologist (term of birth), however in controversial cases it was determined by a neonatologist or pediatrician too, mainly using the Ballard scoring system (6).

The somatometric evaluation was performed using the method of Martin and Saller (7), while the method of individual measurements and normatives were applied according to by Neščáková and Drobná (8). Using the measurements, the precisely defined points published by Borovanský et al. (9), Schumacher (10), Krásničanová and Lesný (11), Bláha and Vígnerová (12) and Čihák (13) should be evaluated. The neonates were examined within 5 days after birth. For these non-invasive measurements, the measuring devices usually applied at the neonatology department, as measuring tape, digital baby scale and a cephalometer, has been used. We followed 16 body parameters, from which we have calculated 5 body indexes.

We have examined the birth weight, birth length, frontooccipital head circumference, maximal length and width of the head, width of the cranial basis, face width (bizygomatic), chest circumference, sagittal and transversal diameter of the thorax, abdominal circumference, biacromial width, bicristal width of the pelvis, maximum arm circumference, maximum femoral circumference and maximum calf circumference. From these measurements we calculated cephalic index (index cephalicus; the ratio of head width to head length multiplied by 100), Quetelet-Kaup index (body mass index; birth weight in grams divided by birth length in centimeters squared), Rohrer-Buffon-Bardeen's index (body massiveness index; the ratio of birth weight in kilograms to birth height in centimeters cube multiplied by 10^5) and thoracic index (the ratio of sagittal diameter to transversal diameter of thorax multiplied by 100). Because of fact that the arm circumference is the somatometric indicator influenced by nutrition of individual and the head circumference is not influenced by nutrition, the ratio of these two factors multiplied by 100 gives the information about nutrition state of an individual (8). It is expressed as the index of arm circumference to head circumference.

The values of body parameters were sorted in groups according to the sex and ethnical origin. For each parameter, the mean value and the Standard Deviation (SD) value have been calculated. Statistical evaluation was performed using the statistical software *SPSS 10.0 for Windows*. Analysis was directed on testing the difference between the group of Romany and non-Romany boys and girls using the method of Student T-test on the level of significance 95% ($p < 0.05$).

RESULTS

Selected body parameters and indexes of Romany and non-Romany neonates are shown in Table 2.

Birth weight, birth length and head and chest circumference are normally used in daily pediatric practice. We have found that Romany neonates are born with significantly lower birth weight, smaller birth length, and smaller head and chest circumference.

Significantly lower values of the following body dimensions were found in both genders of Romany neonates: the width of cranial basis, biacromial width, maximal femoral circumference and sagittal diameter of thorax. In addition, in Romany girls there were significantly lower also the values of abdominal circumference, arm circumference, calf circumference, maximal length of the head and the thoracic index. Romany boys have significantly higher dimension of pelvic bicristal width.

In total, Romany neonates have significantly higher values of cephalic index. They are brachycephalic, while the non-Romany neonates are mesocephalic. Normogram of mentioned values is shown in Figs. 1 and 2.

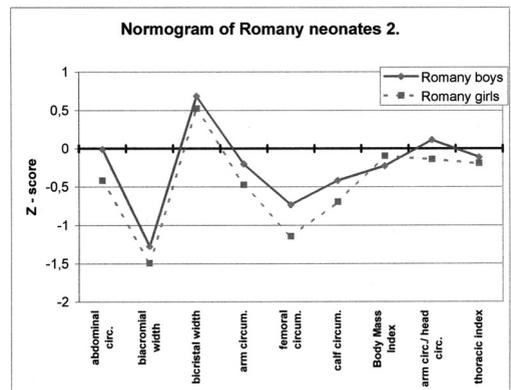
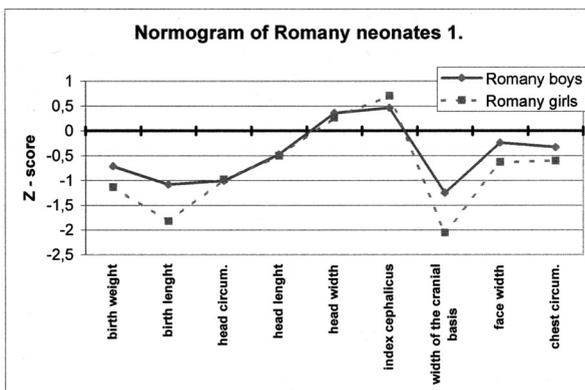


Table 2. : Selected body dimensions and indexes of neonates

	Boys					Girls				
	Romany newborns		Non-Romany newborns		Sign.	Romany newborns		Non-Romany newborns		Sign.
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
Birth weight (g)	3074.34	450.38	3483.31	467.0	p < 0.01	2934.52	455.3	3424.79	373.87	p < 0.01
Birth length (cm)	48.70	2.10	51.09	1.80	p < 0.01	47.45	2.14	50.73	1.53	p < 0.01
Head circum. (cm)	33.56	1.10	34.61	1.09	p < 0.01	32.86	1.34	34.04	1.06	p < 0.01
Head length (cm)	11.66	0.53	11.77	0.43	NS	11.31	0.72	11.59	0.46	p < 0.05
Head width (cm)	9.35	0.29	9.26	0.40	NS	9.27	0.37	9.19	0.36	NS
Index cephalicus	80.25	3.82	78.69	3.40	p < 0.01	82.15	4.26	79.37	3.17	p < 0.01
Width of the cranial basis (cm)	7.78	0.50	8.32	0.47	p < 0.01	7.74	0.44	8.32	0.40	p < 0.01
Facial width (cm)	6.83	0.47	6.90	0.51	NS	6.78	0.76	6.98	0.45	NS
Chest circum. (cm)	32.33	1.62	33.06	1.63	p < 0.01	32.10	1.92	33.03	1.68	p < 0.01
Abdominal circum. (cm)	31.21	1.90	31.67	2.26	NS	30.76	2.24	31.57	1.74	p < 0.05
Biacromial width (cm)	10.45	0.60	11.24	1.07	p < 0.01	10.42	0.74	11.35	1.07	p < 0.01
Bicristal width of pevis (cm)	8.61	0.75	8.38	0.57	p < 0.05	8.48	0.78	8.40	0.65	NS
Arm circum. (cm)	10.20	1.02	10.43	1.00	NS	10.00	0.87	10.46	0.86	p < 0.01
Calf circum. (cm)	10.76	0.99	11.07	0.98	NS	10.47	0.94	10.98	0.89	p < 0.01
Femoral circ. (cm)	15.08	1.41	15.90	1.57	p < 0.01	15.17	1.68	16.71	1.62	p < 0.01
Body mass index (g/cm ²)	1.29	0.12	1.33	0.13	NS	1.30	0.12	1.33	0.11	NS
Arm circum. to head circum.	30.32	2.68	30.13	2.76	NS	30.41	2.29	30.73	2.45	NS
Body massiveness index	2.652	0.025	2.605	0.025	NS	2.718	0.020	2.619	0.020	p < 0.05
Sagit. diameter of thorax (cm)	8.98	0.65	9.18	0.56	p < 0.05	8.75	0.56	9.10	0.59	p < 0.01
Transversal diameter of thorax (cm)	9.86	0.65	9.97	0.62	NS	9.80	0.66	9.94	0.64	NS
Thoracic index	90.94	5.96	92.14	6.16	NS	89.46	4.65	91.84	5.39	p < 0.05

SD – standard deviation; Sign. – significance; NS – non-significant; circum. – circumference; sagit. – sagittal

DISCUSSION

In the care of neonates, nurslings, children and adolescents, it is also important to observe their growth and development. Growth is an especially sensitive indicator of health of the individual, it relates to a large series of biological, medical and socio-economic aspects. That's why it is the basis for the preventive pediatrics (12). In comparison with the majority population, Romany neonates in western Slovakia are smaller in some somatometric parameters that are usually examined in every-day neonatological praxis.

The normal weight of a newborn is 2.500-4.000 g. According to the guideline of WHO, as the low weight at birth the weight under 2.500 g is considered without taking in consideration the length of pregnancy (14). Because of the generally low weight at birth in Romany neonates, confirmed in our research too, in 1976 Bernasovský et al. (15) proposed to create a special limit of low birth weight under 2.250 g for Romany neonates. Bernasovský and Bernasovská (16) support this proposition with the fact that the frequency of full-term Romany neonates with low birth weight, according to today's criteria, is 8.5 %; when using the advised norm it would be decreased to 2.03 %. This percentage still corresponds to the frequency of non-Romany neonates with low birth weight. The proposed criteria are not being used in praxis because of not clear etiology of the difference in weight.

One of the important factors may be the higher smoking in Romany mothers. The mean weight at birth of the smoking mothers neonates is in average 200 g lower than in non-smoking mothers (17). Pathophysiological reason of the negative effect of smoking is insufficient utero-placental circulation (18). The reason is not only the hypoxia of the fetal tissues, but also the insufficiency of vitamins C, B₁₂, folic acid and other substances necessary for the growth and development of the tissues (19). In our study, smoking was declared in more than 15% of Romany mothers. This information was given voluntary, so we can't exclude withholding of the nicotine. According to another study from eastern Slovakia, there were 20.9 % of smoking individuals in non-Romany mothers and 59.6 % in the Romany ones. When presented pregnancy, smoking was left by 70.2 % non-Romany females and only by 52.5 % in the Romany ones (20).

From the aspect of the fetal hypotrophisation, the most important maternal factors are mother's age, parity, possible previous miscarriages, insufficient nutrition, excessive physical exercise, teratogenic effect of medications, as well as narcotics and alcohol (14). The mean age in the Romany mothers was 24.34 years, in non-Romany mothers 30.51 years. In Romany mothers, the parity was nearly twice higher: while non-Romany mothers in our study had on average 1.53 births, the Romany mothers had 3.04 births. The average number of pregnancies in the group of Romany mothers was 3.45. From these data, it is obvious that Romany mothers bear at younger age presented by higher number of pregnancies, which results in shorter periods between the births.

One of the factors resulting in lower values of body parameters in Romany neonates may be a worse socio-economic status of Romany subpopulation. Evaluating the social status we found that 73.2 % of Romany mothers were unemployed or were taking social subvention. 19.7 % of mothers were at maternity leave and only 2.8 % of them were employed. Beside this, when comparing the indexes indicating the nutrition status of neonates (the Body Mass Index and the index of arm circumference to head circumference), we have not found any significant differences (exception is the Body Mass Index, where is significant difference between Romany and non-Romany girls). This fact may prompt of the inadequate use of these indexes in population of neonates or indicate the influence of genetic (ethnic) factors for lower values of body parameters in Romany neonates. Each population has its own growth and weight curves (14). As an example, we can mention the children from India who are smaller at birth in each body parameter. Their place of birth, if it is India, South Africa or Malaysia, is of little importance (16). Several anthropological studies based on follow-up of monogenous and polygenous attributes have confirmed that Romany population has a different genetic outfit (21,22,23). Else Ferák et al. (24) presented data indicating that Slovakian Romanies has the highest coefficient of inbreeding in Europe.

This study may motivate other researches in determining the possible endogenous and exogenous influences on body dimensions and proportions of neonates and may contribute to the diagnostics of hypotrophy in Romany neonates.

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COUGHING AND ITS CARDIOVASCULAR EFFECTS

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Abstract

Cough affects functions of almost all systems and organs including the heart and vessels. Significant changes in basal heart rate and its variability, cardiac output, peripheral and pulmonary circulations occur during coughing. The respiratory sinus arrhythmia is enhanced in dependence on the number and intensity of cough efforts with overall tendency to bradycardia. Effects of the coughing on cardiac functions depend also on the duration of the expiratory phase and a coincidence of the positive pressure with cardiac cycle phases. Remarkable changes in blood pressure and perfusion were found in peripheral, cerebral and pulmonary circulation. Decrease of the cerebral perfusion during prolonged cough attacks may cause posttussive or cough syncope. To the mechanism of the syncope are involved many (mechanical, neural and humoral) factors.

Voluntary cough (cough - cardiopulmonary resuscitation - c-CPR) can be used in some specific indications, to maintain sufficient blood flow to the brain and to keep the heart and brain functioning for a short time.

Key words: cough, cardiovascular system, respiratory sinus arrhythmia, cough syncope, cough self-resuscitation

INTRODUCTION

Coughing as the most important defensive airways and lung reflex (1) is accompanied with strong specific activation of nervous system (receptors, relevant functional structures in brain, etc.) and forceful respiratory muscles activity with primary mechanical effects and secondary consequences. Cough significantly affects functions of almost all systems and organs including the heart and vessels.

The various cardiovascular parameters can be modified in all phases of cough - before, during and after coughing. *Before* the cough, there are activation followed by inhibition and vice versa of variety of receptors, neurons and structures of the nervous system. *During* coughing are dominant pressure fluctuations, and *after* there are results the autonomic nervous system (ANS) activity changes, blood gases effects and others. There is an exceptional huge complex of effects with their specific responses in the cardiovascular system.

1. VENOUS RETURN CHANGES

In *the deep preparatory inspirium* intrathoracic pressure falls to the values -15 up - 35 mmHg, and intraabdominal pressure (permanently positive during quiet breathing), conversely rises by a deeper descent of diaphragm. The increased pressure gradient between abdominal and thoracic cavities - respiratory thoracico-abdominal pump - helps to the venous return because backflow is prevented by venous valves in the leg veins.

The pumping effect in the very deep inspirium can be limited by partial collapse of the vena cava in diaphragm (2). However we suggest this partial obstruction does not represent only a limitation of the physiological process. It is possible that this partial obstruction can play a physiological role. Limitation of the blood inflow can protect against an excessive aspiration of blood to the right atrium and through this mechanism it can defend the right ventricle from overloading.

In the following phase characterized by *the active expiratory effort*, the maximum pleural pressure reaches up 200 mmHg in healthy subjects (3). Also the intraabdominal pressure increases in this active expiratory phase by abdominal muscles contraction. The intraabdominal pressure attaining high values during cough is transmitted to the intraabdominal vessels and to the pelvic organs. During the expiratory cough efforts when both pressures are high, the venous return from abdominal veins will depend again on a gradient between the intraabdominal and intrathoracic pressure. Intraabdominal pressure can exceed intrathoracic only when lung vol-

umes are small (coughing from functional reserve capacity - FRC – level). The expiratory effort is performed in this situation predominantly by abdominal muscles (4) and venous return is enhanced in this phase. In other situations the venous return is diminished.

2. CARDIAC ACTIVITIES

Changes in heart rate and its variability

Heart rate is under the regulatory effects of autonomic nervous system which tonic discharges are modified by reflex mechanisms. Beside the nervous regulation, including baroreflexes, heart rate is responding very sensitively to local pressure and temperature, distention of the right atrium (Bainbridge effect) due to venous return changes and to endocrine glands activities.

Instantaneous heart rate (beat-to-beat; RR intervals) fluctuates promptly in relations to breathing, changes in blood pressure via baroreflexes, and to other factors. The effect of the quiet breathing in humans can be seen on ECG records as *the respiratory sinus arrhythmia*. During coughing, the respiratory sinus arrhythmia is enhanced in dependence on the number and intensity of the cough efforts, fluctuations of negative / positive intrathoracic pressure and on the following blood pressure changes.

We have studied **the heart rate variability** during and after cough attack in experimental animals (5,6) and in healthy volunteers (7).

In the experiments on anaesthetized cats, the positive intrathoracic pressure during the expiratory phase with concomitant systemic arterial pressure changes caused an enhancement of the heart rate oscillations and an overall tendency to bradycardia. After the cough attack, a significant increase in the heart rate simultaneously with a hypotensive reaction occurred.

In humans, after a voluntary single cough performed from the functional residual capacity (FRC) and total lung capacity (TLC) level, RR intervals became significantly shorter. Peak of the tachycardia was between 4th - 5th RR interval. The increase in heart rate started earlier (deep inspiration), it was greater and lasting longer in cough from TLC than from FRC.

The short-term variability of the heart rate, we have studied also by means of system VarCor PF6. The tachycardic response was present even after one cough, three cough efforts were accompanied by a prolonged positive chronotropic response after cough attack.

Changes in ECG

Changes of ECG associated with mechanical irritation of the airways were described, as **arrhythmia, extrasystoles** of various type, **atrioventricular blocks, ischemic signs** of ST-segment, T-wave etc. (8,9). These changes during cough and the Valsalva's maneuver occurred more frequently in patients with the ischemic heart disease and may be ascribed to hypoxia.

In our experiments on anaesthetized cats, the defensive reflexes were accompanied only very rarely, in 5-15 % of stimulations, with pathologic ECG changes. Also in our observations in healthy volunteers, coughing was not accompanied with pathological ECG changes.

Variations in cardiac output

Coughing by its powerful mechanical actions can produce changes in stroke volume and cardiac outputs. These variations may be in both ways – in improving or worsening of the pumping effectivity depending on the venous return, blood flow through pulmonary circulation, or on a coincidence of the intrathoracic pressure with phases of cardiac cycle.

During *the deep preparatory inspiration*, end diastolic volume (EDV) in the right ventricle (RV) increases together with the enhancement of the respiratory pump effect on venous return, and as follows greater right ventricle stroke volume and blood flow in pulmonary artery. The greatest stroke volume was found at the end of the deep inspiration (10).

Short-lasting and high positive intrathoracic pressure *during expiratory phase* of coughing acts on all structures inside the thorax. If the ventricles eject the blood in the same time, and the increase in intrathoracic pressure is sudden, intensive at the beginning of the ejection phase of the cardiac cycle, the effect is in summation of the involved pressures. Intrathoracic pressure is transmitted to the heart cavities and the contractile force produced by the myocardium is

enhanced. The stroke volume and pumping efficiency of the ventricles can be improved by 25-50 % (11,12). On the other side, the positive intrathoracic pressure squeezes intrathoracic vessels, increases afterload of the right ventricle and diminishes the pumping effect.

Final effect of the positive intrathoracic pressure on cardiac functions depends also on the height of the pressure, duration of the expiratory phase, velocity of the pressure changes and on the mentioned coincidence of the positive pressure with cardiac cycle phases.

3. PERIPHERAL CIRCULATION

In spontaneously breathing subjects, coughing results in considerable oscillations of peripheral blood pressure although there is an overall tendency to hypotension. These changes are enhanced in prolonged cough seizure, when many times repeated positive efforts reduce venous return, central blood volume and cardiac output. This is why during cough attack, mainly if the expiratory efforts are dominant over the inspiratory can occur tendency to decreases in blood pressure and flow in aorta as well as in other peripheral arteries.

During the cough attacks, also in our experiments on spontaneously breathing cats, the considerable inspiration-expiration oscillations of the intrathoracic pressure were mechanically propagated to the blood circulation, thus leading to the marked oscillations of the blood pressure in systemic circulation - to the sudden elevations of the systolic and decreases of diastolic blood pressure in femoral artery with enhanced pressure amplitude.

The sudden changes of intrathoracic and intraabdominal pressure are transmitted also to the peripheral venous system, where they cause considerable and rapid changes in blood pressure. These rapid changes of blood pressure and flow in the veins and in the heart cavities may cause a release of venous thrombi with a subsequent embolism. Mean blood pressure in venous peripheral system shows a tendency to an elevation what can be a manifestation of the venous return impairment and stagnation of the blood in the veins.

The typical changes in acral circulation - in blood pressure registered by Finapres device (Ohmeda, U.S.A) together with a photoplethysmographic curves (ELCAT, Germany) monitoring blood perfusion in fingers of left hand in a man are in the **Fig. 1** and **2**. Each of the cough efforts is transmitted up to the finger circulation resulting in the increase of the monitored parameters during a cough.

Posttussive reaction: After a cough attack, systemic blood pressure significantly decreases. The hypotensive reaction is present also in the acral circulation.

Heart rate and blood flow in abdominal aorta, or in femoral artery rise after coughing. The increase in blood flow can be due to improvement of the venous return by mobilisation of accumulated blood in caval system followed by changes in stroke volume and cardiac output. To this improvement of the peripheral perfusion after coughing is involved also a decrease of peripheral resistance.

The purpose of the peripheral hyperperfusion is an improvement of the oxygen and nutrients delivery to tissues and organs which suffered by their lack during cough attack.

Cerebral circulation

Remarkable changes in blood pressure and perfusion were found in cerebral circulation during the cough.

Systolic *blood pressure* in common and internal carotid arteries rises sharply in expulsive phase of coughing, but diastolic pressure usually remains unchanged (13). Despite this rise in systolic pressure, *blood inflow* to the cerebral circulation diminishes. A cause of this paradox lies in the intracranial pressure changes.

The cerebral circulation has a special protective mechanism: the rise in *intracranial pressure* compresses the vessels from outside. It is functioning as a very important mechanism against overdistention of the cerebral vessels, rupture and cerebral apoplexy. Through the increase of vessels stiffness, coughing does not provide rising the transmural pressure to a dangerous level. Small cerebral arteries, arterioles, capillaries can collapse during high values of the intracranial pressure because their blood pressure is lower physiologically.

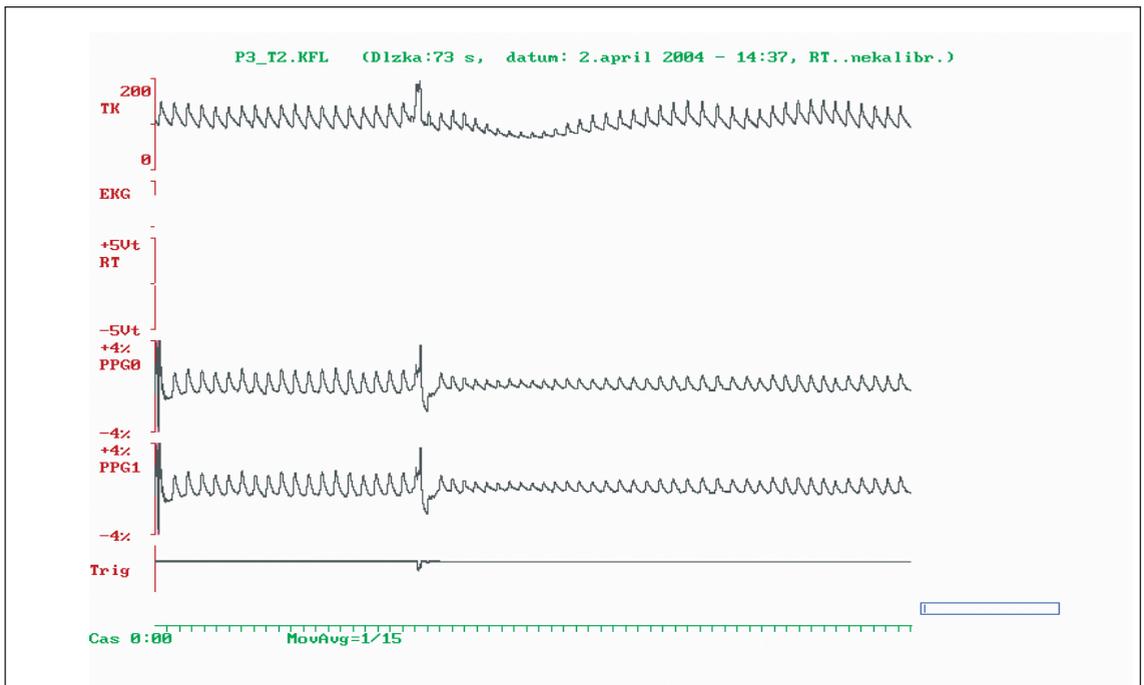


Fig. 1 Acral blood pressure (TK – upper trace) and photoplethysmographic curves (PPG0, PPG1) before, during (signal in Trig) and after one voluntary cough effort from the TLC level.

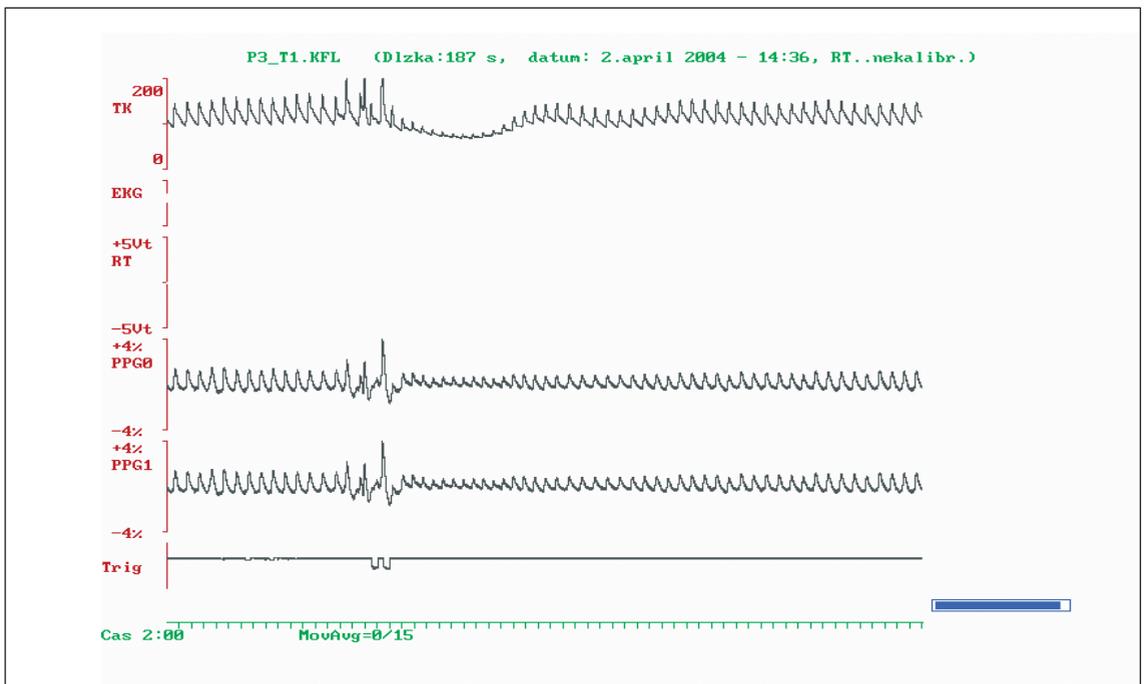


Fig. 2 Acral blood pressure (TK – upper trace) and photoplethysmographic curves (PPG0, PPG1) before, during (signal in Trig) and after three voluntary cough effort from the TLC level.

If a cough attack lasts longer, systolic and diastolic pressures in the carotid arteries begin to decline, together with cardiac output. *Cerebral perfusion* diminishes and it can lead up to the cerebral hypoxia.

The decrease of blood pressure and cerebral perfusion during intensive and prolonged cough attack may cause *posttussive or cough syncope* (1,14,15.). The cough syncope can be seen mainly in middle-aged men in whom the intrathoracic pressure can reach up very high positive level. The predisposed subjects have disorders of vision at culmination of the cough seizure and they lose temporarily consciousness in the following 1-2 s. In the same time, together with a loss of consciousness, the coughing disappears. The attack can be accompanied by clonic convulsions. Consciousness returns back in approximately 10 seconds, the whole episode lasts about 30 seconds.

The main mechanisms of the cough syncope are cardiovascular changes and intermittent cerebrovascular insufficiency caused by a decrease in the cerebral blood flow, when it cannot be elevated to the values necessary to cover brain metabolic needs. In the development of the cough syncope, more factors can contribute, like peripheral vasodilation, collaps mechanism limiting venous return, reflex bradycardia, pulmonary vasoconstriction, decrease in cardiac output, as well as the elevation of intracranial pressure. The changes in blood gases during coughing can play also a role in the development of cough syncope. Coughing as a short hyperventilation can be accompanied by hypocapnia (16). A fall in paCO_2 has a constrictor effect on cerebral vessels, and cerebral vasoconstriction can be an important factor in the production of the cough syncope.

4. PULMONARY CIRCULATION

Pulmonary circulation in coughing is affected mechanically due to its location in the thorax and other mechanisms. Systolic *blood pressure* in pulmonary artery during the deep preparatory inspirium decreases, in the expiratory phase it rises in dependence on the intrathoracic pressure. Mean blood pressure in pulmonary artery during coughing falls, mainly in the cases with a predominance of the inspiratory efforts.

The pulmonary vascular resistance rises during coughing in cats not only in the expiratory but also in the inspiratory phase proportionate to lung volume Samseli et al. (10). This phenomenon can be explained by lengthening and narrowing of the pulmonary vessels.

Blood flow through pulmonary artery is different in various phases of cough in a dependence on many factors. However, the average value of the pulmonary blood flow during the whole cough attack decreases.

During tracheobronchial coughing, when inspiratory efforts are not so deep, the blood flow through pulmonary artery in the expiratory phase is increased. Blood is squeezed to the left atrium and to the left ventricle in expiratory phase, and central blood volume lowers. In prolonged cough attack, the resistance against the inflow from the right ventricle is increased and flow to the left atrium diminishes sharply.

5. CLINICAL IMPLICATIONS

Recently, we do not know all details about possible *positive cardiovascular effects* of the coughing. We are sure, that by the same way, as an enhancement of the heart rate variability in deep breathing (RSA) helps to coordinate activities of the respiratory and cardiovascular system to better oxygenation (17,18), the cardiovascular effects of coughing are not only passive consequences, but they have their important physiological role.

A suggestion, how to utilize voluntary cough and concomittant cardiovascular changes for cardio-pulmonary-cerebral self-resuscitation – „cough CPR“, have been presented by Petelenz et al., (19,20). They suggest that during a sudden arrhythmia, it may a conscious subject to cough forcefully and maintain sufficient blood flow to the brain, it may also rectify the heart rhythm and keep the heart and brain functioning longer time (approximately 10 min).

The idea is not entirely new. Criley et al. (21) described successful resuscitation of patients undergoing coronary angiography from ventricular fibrillation. They used coughing every one to three seconds. The mean aortic pressure induced by cough was nearly 140 mmHg and only 60 mmHg by external CPR.

Cough – CPR supports circulation by compressions of the heart provided by respiratory muscles through intermittent and high positive intrathoracic pressure (internal cardiac massage) and cardiovascular consequences of the coughing. This technique of coughing to maintain blood flow during brief periods of arrhythmias needs more study. However it has been found to be useful in the hospital, particularly during cardiac catheterization, when ECG is monitored continuously.

In conclusion, as follows from the analysis, the cardiovascular effects of coughing are complex processes. Study of different factors and their role in the development of cardiovascular effects during coughing will enable not only better understanding the physiological mechanisms, but rational prevention and treatment of their disorders as well.

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INFLUENCE OF DISTAL ESOPHAGEAL ACIDIFICATION ON COUGH SENSITIVITY IN HEALTHY VOLUNTEERS

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Abstract

Chronic cough is commonly associated with gastro-esophageal reflux (GER). The mechanisms underlying GER-related cough sensitivity in humans are not fully understood. The aim of our study was to evaluate the effect of distal esophageal acidification (Bernstein test) on the cough sensitivity in healthy volunteers.

Single-breath capsaicin test was used to assess cough sensitivity. Capsaicin aerosols of doubled concentrations from 0.49 to 500 $\mu\text{mol.l}^{-1}$ were inhaled to the concentration that elicited coughing. This concentration of capsaicin represented the cough sensitivity (CS). Inhalation cough challenge with capsaicin was performed in 18 healthy volunteers (10 M, 8 F; mean age 22 yrs.). A nasogastric catheter was introduced into the distal esophagus and esophageal mucosa was perfused with 150 ml of saline during 15 minutes. CS was assessed immediately after distal esophageal perfusion with saline. Control saline perfusion was followed by distal esophageal acidification with 150 ml of 0.1 mol.l^{-1} HCl during 15 minutes. CS test was performed after Bernstein test again.

15 minutes lasting distal esophageal acidification was not accompanied by heartburn and /or coughing. Measurements of capsaicin cough sensitivity (geometric mean, 95% confidence interval) were not significantly lower after distal esophageal acidification compared to physiological saline distal esophageal perfusion [13.4 (3.7 – 48.0) $\mu\text{mol/l}$ versus 11.9 (3.6 – 40) $\mu\text{mol/l}$; $p=0.51$].

The results indicate that distal esophageal acidification (lasting 15 minutes) does not influence cough sensitivity in healthy volunteers.

Key words: cough, cough sensitivity, gastro-esophageal reflux, Bernstein test

INTRODUCTION

Chronic persistent cough has been the most widely studied entity in the investigation of the role of gastro-esophageal reflux disease (GERD) in the pathogenesis of cough. It is defined as cough persisting for at least 3 weeks in patients with a normal chest X-ray and not on angiotensin-converting enzyme inhibitors. In multiple series, gastro-esophageal reflux (GER) has been documented to be a cause of chronic persistent cough (either solely or in combination with bronchial asthma and postnasal drip) in 38-82 % of patients (1-7).

The pathophysiologic mechanisms underlying GER-related cough are not fully understood but may include microaspiration of esophageal contents into the larynx and tracheobronchial tree or a vagally mediated esophageal-tracheobronchial reflex (8, 9). A self-perpetuating cough-reflux cycle has been proposed in which esophageal acid stimulates cough, and cough, in turn, amplifies reflux by increasing transdiaphragmatic pressure (5, 8).

The primary event in GER is the movement of gastric contents into the esophagus. There are several possible mechanisms whereby impaired gastric emptying could contribute to GER-related cough. A delay in emptying could cause gastric distension, which has been shown to increase the rate of transient lower esophageal sphincter relaxations. Second, impaired gastric emptying may lead to slow clearance of esophageal acid, which has been implicated in the pathogenesis of some cases of chronic cough (9, 10).

Chronic cough and asthma are two clinical problems caused or triggered by GER disease (1). Irwin et al (1) indicated chronic persistent cough correlated with distal but not proximal acid reflux events usually showing evidence of distal esophagitis observed with endoscopy; they con-

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cluded that acid stimulated inflamed distal esophageal mucosal receptors, resulting in a reflex-mediated cough. Effect of GER on cough responsiveness in patients with bronchial asthma was studied by Wu et al. (11). They concluded that acid stimulation of the lower esophagus increased cough responsiveness to capsaicin in patients with mild persistent asthma, even in those without GER symptoms or evidence of esophagitis.

The aim of our study was to evaluate the influence of distal esophagus acidification (Bernstein test) on cough reflex sensitivity in healthy volunteers. The mechanisms by which cough sensitivity is influenced or triggered by gastro-esophageal reflux are still not clear.

METHODS

Subjects

The study population consists of 18 healthy volunteers (10 M, 8 F; mean age 22 yrs.) with no clinical respiratory symptoms, normal spirometry and no history of acute respiratory infection (last 6 weeks) who were recruited consecutively. They have no history of respiratory, allergic, endocrine and cardiovascular diseases. Smokers were also excluded from the study.

Based on a structured, interviewer-led questionnaire, each subject was asked about respiratory symptoms and a past and family history of bronchial asthma, allergic rhinitis, gastro-esophageal reflux, cardiovascular diseases, metabolic diseases, ACE inhibitor treatment.

The study was approved by Ethics Committee of Jessenius Medical School and informed consent was obtained from all subjects after the purpose of the test had been explained.

All patients were examined at the same time of day. Subjects attended the laboratory to undergo a distal esophageal acidification, cough sensitivity test and spirometry.

Cough Inducement Test

Measurement of cough threshold to inhaled capsaicin (pungent extract of red pepper stimulating vanilloid receptors) was carried out using the modified method of cough response test by capsaicin inhalation originally reported by Dicipinigaitis and Rauf (12). Each subject inhaled an aerosol of control solution (physiologic saline), followed by progressively increasing concentrations of the capsaicin solution (SIGMA) (from 0.49 to 500 $\mu\text{mol.l}^{-1}$). Aerosols were inhaled every 60 s wearing a nose clip from a nebuliser ProvoJet (Ganshorn Medizin Electronic, Germany). The number of capsaicin-induced coughs during the first 30 s after each dose was counted. The inhalation challenge was performed immediately after esophageal HCl or saline solution perfusion. Subjects were unaware that cough was a specific point of research interest.

Results were expressed as the lowest concentration of capsaicin causing two or more coughs. Capsaicin cough threshold, the lowest concentration of capsaicin eliciting two or more coughs, was measured as an index of airway cough sensitivity.

Acid Provocation Test

Esophageal acid provocation was performed in a sitting position. A nasogastric catheter was introduced transnasally into the distal esophagus. Through the esophageal catheter, the outlet of which was positioned at approximately 15 cm above the lower esophageal sphincter, 0.1 mol.l^{-1} HCl was perfused at a rate of 10 ml/min for 15 minutes. Cough sensitivity was obtained by capsaicin inhalation test with the catheter in the esophagus. The catheter was firmly set and not removed during the cough test.

One week later, cough test was performed again in similar method, except with saline solution perfusion instead of esophageal HCl perfusion (although the order of HCl and saline solution perfusion was done at random). In the present study, the patients were unaware of whether HCl or saline solutions was perfused.

Data analysis

The capsaicin cough threshold was expressed as geometric mean value with 95 confidence intervals (CI) of the capsaicin concentration causing two or more coughs. Geometric mean and 95 % CI were calculated for saline (control) and acid perfusion of distal esophagus. Data were

analysed by non-parametric analysis of variance (Kruskal-Wallis' test), a value of $p < 0.05$ was considered to be significant.

RESULTS

15 minutes lasting distal esophageal acidification was not accompanied by heartburn and/or coughing. Cough sensitivity expressed as geometric mean (95% CI) of capsaicin concentration was 11.9 (3.6 – 40) $\mu\text{mol/l}$ in 18 healthy subjects after control esophageal perfusion with physiological saline and 13.4 (3.7 – 48.0) $\mu\text{mol/l}$ after distal esophageal acidification with 150 ml of 0.1 mol.l⁻¹ HCl during 15 minutes ($p = 0.51$). Distal esophageal acidification (lasting 15 minutes) does not influence cough reflex sensitivity in healthy volunteers (Fig. 1). In addition, none of the patients expressed any different feelings between HCl or saline solution perfusion in the esophagus.

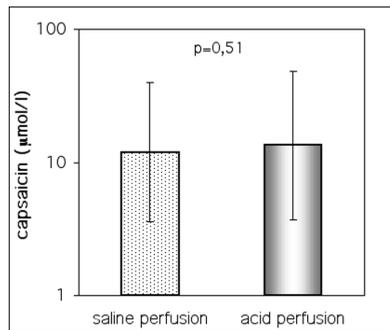


Fig. 1 Cough sensitivity expressed as geometric mean and 95 % CI of capsaicin concentration inducing 2 or more coughs after saline and hydrochloric acid distal oesophageal perfusions in healthy volunteers

DISCUSSION

The aim of our study was to evaluate the effect of distal esophageal acidification (Bernstein test) on the cough sensitivity in healthy volunteers. Distal esophageal acidification (lasting 15 minutes) does not influence cough sensitivity in healthy volunteers.

We have shown in this study that the instillation of hydrochloric acid into the distal esophagus of healthy volunteers does not trigger the cough. This suggests that the distal esophagus of healthy volunteers is not hypersensitive to various stimuli, including acid and possibly physical agents, such as cool isotonic saline.

Wu et al. (11) demonstrated that distal esophageal acid perfusion itself can increase cough responsiveness in patients with mild persistent bronchial asthma even without GER symptoms and the presence of esophagitis. It was previously shown that chronic persistent cough that remains unexplained after a standard diagnostic evaluation is associated with either asymptomatic GER (1, 5) or impaired clearance of acid from esophagus (13).

Irwin et al. (1) indicated cough correlated with distal but not proximal acid reflux events, with endoscopy, usually showing evidence of distal esophagitis. They concluded that acid stimulated inflamed distal esophageal mucosal receptors, resulting in a reflex-mediated cough. Ing et al. (8) suggested that acid in the distal esophagus precipitates cough, and that there is evidence for an esophageal-tracheobronchial cough reflex mechanism in patients with chronic cough associated with GER. However, their subjects were not asthmatic patients. In study of Wu et al. (11), the patients with mild persistent bronchial asthma have neither GER symptoms nor esophagitis, which was evaluated by gastroesophagoscopy.

There are two proposed mechanisms of GER-associated cough: 1. acid in the distal esophagus stimulating an esophageal-tracheobronchial cough reflex, and 2. microaspiration or macroaspiration of esophageal contents into the larynx and tracheobronchial tree (importance of recumbent position, sleep and deep inspiration before cough). A self-perpetuating cough-reflux cycle has been proposed in which esophageal acid stimulates cough, and cough, in turn, amplifies reflux by increasing transdiaphragmatic pressure (5, 8). We studied the artificial GER-associated cough responsiveness while acid in the distal esophagus stimulated an esophageal-tracheobronchial cough reflex, though the mechanisms would need to be elucidated further.

Hamamoto et al. (14) studied the airway plasma extravasation induced by intraesophageal HCl stimulation in anesthetized guinea pigs, and found that infusion of 1 N HCl into the esophagus significantly increased plasma extravasation in the trachea, which was inhibited by capsaicin or bilateral vagotomy. They thus concluded that tachykinin-like substances are released to cause plasma extravasation in the airways as a result of intraesophageal HCl stimulation, and there are neural pathways communicating between the esophagus and airways, including the vagus nerve. Capsaicin releases tachykinins from storage in nerve endings in the airway. Substance P is one of the potent tachykinins to stimulate C fiber and to induce cough. The increased capsaicin-induced cough sensitivity by HCl perfusion of lower esophagus was due to the increased susceptibility of C fiber-mediated vagal nerve network communicating between airway and esophagus (15). In the present study, spontaneous coughing did not occur during the acid stimulation to esophagus on all subjects. Therefore, the acid stimulation to esophagus itself neither increased cough sensitivity to capsaicin, nor induced spontaneous cough.

In this regard, GER may increase cough responsiveness when asthmatics receive stimuli to their airway (11). There is no report concerning the effect of HCl perfusion on cough sensitivity of normal subjects. However, Schmidt et al. (16) reported that in normal subjects and in patients with mild bronchial asthma, cough thresholds were not significantly different from each other.

The present data suggest that artificially induced GER does not lead to an increase in cough responsiveness in healthy volunteers. The unchanged cough sensitivity is a result of unrepresented esophagitis and/or airway inflammation in healthy volunteers or stimulation of esophageal mucosal afferent nerve-endings is unable to change the cough sensitivity.

We conclude that acid stimulation of the lower esophagus does not increase cough responsiveness to capsaicin in healthy volunteers. The mechanisms responsible for induction of cough during distal esophageal acidification in patients with chronic cough need further investigation.

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VIDEO-ASSISTED THORACOSCOPIC LOBECTOMY. IS IT AN ADEQUATE PROCEDURE FOR TREATMENT OF LUNG CANCER?

Case report

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Abstract

Video-assisted thoracic surgery (VATS) in major pulmonary resection is emerging as a potential alternative to open surgical approach for early stage lung cancer. However, the implementation of VATS has been limited, partly due to concerns about its effect on local control of tumor, its recurrence and long-term survival. Reported series of patients undergoing VATS lobectomy for pulmonary malignancy have not been able to respond questions of safety of procedure and its oncological adequacy because they had only short-term follow-up. That is why VATS lobectomy has remained infrequent despite its technical feasibility.

This case report presents personal experience with two VATS resections in lung cancer patients, discusses technique, indications and limitations of procedure and reviews the results reported in literature with emphasis on its controversies.

In conclusion the authors do not consider VATS lobectomy for clinical stage I primary lung cancer as an inappropriate procedure. However, currently they recommend open approach as the gold standard, before randomised long-term follow-ups will prove comparable benefit of VATS lobectomy on survival.

Key words: video-assisted thoracic surgery (VATS), VATS lobectomy, lung cancer

INTRODUCTION

There is no doubt, that complete surgical resection remains the method of choice in patients with resectable lung cancer. During the last decade of 20-th century a significant promise has been shown in operative technique offering a less invasive but potentially as effective approach as thoracotomy. Despite the videoendoscopic revolution in surgery after the success of laparoscopic cholecystectomy in the 1980s, that now affects all surgical disciplines, video-assisted (VATS) pulmonary lobectomy as a cancer operation is still controversial. Although recent studies have shown its technical feasibility and some benefits compared to lobectomy by thoracotomy (Table 1), these studies failed to prove any superiority for the VATS approach in patients with lung cancer (1,2,3). There are concerns about safety of VATS lobectomy, its effect on tumor recurrence and survival of patients undergoing VATS resection for pulmonary malignancy. That is why the most thoracic surgeons are still reluctant to use it and, as a result, it is adopted only in certain thoracic centres.

Table 1. Advantages and disadvantages of VATS lobectomy.

ADVANTAGES	DISADVANTAGES
less postoperative pain	lack of manual palpation
reduced release of tumoral mediators	risk of serious bleeding
better preservation of lung function risk	of residual cancer
better cosmetic effect	risk of prolonged air-leak
shorter hospital stay	risk of conversion to thoracotomy
earlier recovery	? long-term survival?

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INDICATIONS AND CONTRAINDICATIONS FOR VATS LOBECTOMY

The indications for VATS lobectomy are similar to indications for conventional resection, but because of concern regarding the ability to perform a complete resection and a proper lymph node dissection with minimal access, only early primary lung cancer without mediastinal lymph node involvement is recommended for VATS lobectomy. For the same reason a preresectional lymph node staging including mediastinoscopy is mandatory. The question of safety is associated with central tumors involving hilar structures of lung, where dissection of lobar vessels in an closed chest could carry a potential risk of serious bleeding. Therefore, central tumors greater than 5 cm in diameter should be excluded from endoscopic approach. Absolute contraindications to VATS lobectomy are extensive pleural adhesions, chest wall involvement of tumor, severe bleeding disorder and the inability to tolerate single lung ventilation.

OPERATIVE TECHNIQUE

VATS technique is advisable only to a surgeon who is experienced in open thoracic surgery. Besides a new endoscopic skills it demands good hand-eye coordination and familiarity with more sophisticated endoscopic technology and instruments. The need of general anesthesia with single lung ventilation and patient positioning at a full lateral decubitus position are the same as for open thoracotomy approach, to which the surgeon should be prepared for, when technical problems arise. Two main 10-12 mm operating channels are created for introducing the thoracoscope and endoscopic instruments or stapler. Most surgeons perform so-called "utility minithoracotomy" (generally 4 to 6 cm in length) at the beginning of the procedure, which is placed over the 4th intercostal space and provides an easy direct access to the hilar structures. Minithoracotomy is also used for extraction of the resected lobe. A rib spreader is not recommended, but a soft tissue retractor for the minithoracotomy wound could be useful. Hilar dissection is performed by means of endoscopic or conventional instruments and stapler transection or hand ligation with extracorporeal knots are the most frequent methods of interruption of hilar vessels. Bronchus is routinely managed with stapler. After a retrieval of involved lobe, two silicone chest drains are placed through the working channels and minithoracotomy is closed.

CASE REPORTS

The first patient was a 61-year-old male presenting with hemoptysis. After completion of preoperative examinations including mediastinoscopy a peripheral right lung nodule remained to be undiagnosed and the patient underwent videothoracoscopic procedure. Right lower VATS lobectomy was performed on the basis of intraoperative frozen section of the tumor, which revealed combined adenosquamous carcinoma. The postoperative course was uneventful and patient was discharged from the hospital 11 days after the operation. Six cycles of adjuvant chemotherapy were employed according to the final pathohistological examination of the operation specimen which revealed *pT2N1M0* stage of lung cancer. He was regularly followed up and during the last control (5 years after VATS lobectomy) no signs of recurrence were detected.

The second patient was a 45-year-old male complaining of hemoptysis and chest pain. A CT scan of the thorax confirmed tumor-like lesion located in the periphery of the left lower pulmonary lobe. Bronchoscopy and fine needle aspiration revealed no malignant disease. Patient was submitted to Carlens mediastinoscopy and pulmonary resection as the one-stage procedure. After negative mediastinoscopy left lower VATS lobectomy was performed. Histological examination revealed a squamous cell carcinoma in stage *pT2N0M0*. The patient had a smooth recovery and he was discharged 8 days postoperatively. On follow up consultation five years after surgery he was asymptomatic, living an active life.

DISCUSSION

Despite a very good experience with the first two cases of VATS lobectomy, which were performed in 1995 as the pioneering video-assisted major lung resections in Slovakia, the authors agree with the others who consider VATS lobectomy for pulmonary malignancy controversial.

VATS lobectomy is not appropriate for every patient and even for every surgeon. The surgeon should be experienced in VATS before attempting endoscopic lung resection. It is generally believed that palpation plays an important role in the assessment of the tumor mass, and its invasion to other structures. This can not be used in case of VATS and in addition, the palpation of the remaining lung for occult tumor nodules is not possible either.

Major complications resulting from VATS lung resection are relatively uncommon. Persistent air-leak beyond several days is the most common cause of postoperative morbidity. There is some evidence that VATS presents a considerably lower rate of morbidity than thoracotomy and this is related to the reduced level of invasiveness required for endoscopic access (4,5). Massive bleeding from dissected pulmonary vessels appears quite rarely and skillful endoscopic surgeon should know how to prevent or manage it. The incidence of serious bleeding during a VATS lobectomy appears to be very low (0.3 %) in the present series of patients (6). The VATS technique has been shown to be safe and there are documented some advantages over the thoracotomy in terms of less postoperative pain, earlier recovery and better preservation of postoperative lung function. However, the problems of pain related morbidity, the chest tube duration and the length of hospital stay, which were less frequent after VATS lobectomy, did not reach statistical significance and neither proved any significant benefit to the VATS approach by randomized prospective studies (7,8). Moreover, these advantages appear to lose effect within a few weeks after surgery. On the other hand, surgeons who still prefer an open approach, perform currently limited muscle-sparing thoracotomies and use epidural analgesia to minimize thoracotomy related pain. It is also important to point out that there are several variations of VATS technique which is not unified.

The main criticism regarding the use of VATS in major pulmonary resection concerns the indications for this type of procedure, with particular reference to compliance with the principles of oncological surgery. These principles direct the surgeon to a careful selection of patients, in order to operate on stage I patients only, preferably with squamous type tumor (9) and localised in a peripheral part of the lung. On the basis of these considerations it may be stated that VATS technique is indicated for relatively small group of patients, in which a conventional approach can be accomplished through a limited muscle-sparing thoracotomy without any significant differences in morbidity and mortality.

Survival data following VATS lobectomy have been presented by various authors although usually with limited follow-up (1,10,11,12). These reports describe results which are comparable with ones achieved by conventional approach, but at relatively short follow-up intervals (Table 2). Therefore randomised studies are still required to confirm or to moderate some enthusiastic claims on a possible priority of VATS approach over thoracotomy.

Table 2. VATS lobectomy-multicentric retrospective review-McKenna,1998 (1)

Number of patients	298
Postoperative morbidity	12.8 %
Postoperative mortality	0.3 %
Conversion to thoracotomy	6 %
Median length of hospital stay	4 days
4-year survival for stage I NSCLC	70 %

CONCLUSION

Videothoracoscopy have been proven as an excellent therapeutic method for a various benign intrathoracic diseases, but there are still some issues of its safety and oncologic efficiency, which

should be analysed before recommending this procedure for widespread use in lung cancer. The aim of lung cancer resection is to perform a complete removal of the tumor along with all regional lymph nodes, which can provide long-term cure. This can be managed properly by open approach, which is not a compromise operation with minimal morbidity and short term benefit.

VATS approach seems to be a valid alternative to conventional open procedure only for selected group of patients with stage I lung cancer if complete resection is feasible. However, majority of thoracic surgeons consider thoracotomy as the gold standard and the application of VATS lobectomy has remained infrequent. Obviously, any new procedure must achieve morbidity, mortality, and long-term survival rates equivalent to those of conventional approach. Until these results are not achieved the dilemma can be expressed by the statement of the famous surgeon Belsey: „The battlefields of surgery are strewn with the remains of promising new operations which perished in the light of the follow-up clinic“.

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