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ASTHMA BRONCHIALE PHENOTYPES AND THEIR TREATMENT – A CURRENT VIEW

¹VRLIK M., ²DZURILLA M., ² BUCOVA M., ^{1,3}KANTAROVA D., ²BUC M.

¹ Martin Immunology Centre, Martin, Slovakia

 ² Department of Immunology, Comenius University School of Medicine, Bratislava, Slovakia
 ³ Department of Internal Medicine 1, Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic

Abstract

Asthma bronchiale is an immune-mediated disorder of the conducting airways and lung parenchyma, which is characterised by periodic, reversible inflammation and constriction. It develops on the intersection of a genetic predisposition and environmental factors, which start a complex of immunopathological reactions resulting in clinical manifestations. Recognition of the cellular and molecular mechanisms of allergic reactions enabled to subdivide clinical forms of the disease into four different phenotypes, eosinophilic, neutrophilic, paucigranulocytic, and steroid-resistant, respectively. Moreover, it helped to identify new targets for biological therapy.

Key words: asthma bronchiale, phenotypes, immunotherapy, pharmacotherapy

INTRODUCTION

Asthma bronchiale is a chronic inflammatory disease derived from airway inflammation and broncho-constriction. Basically, there are two forms of asthma, allergic or non-allergic. The pathology of non-allergic asthma appears very similar to that of allergic asthma, although there have been some differences (1). Allergic asthma develops in individuals with a genetic predisposition to the disease when environmental factors launch a complex of immunopathological reactions. Allergen exposure results in activation of numerous cells of the immune system. Previously asthma was considered purely as a Th2 disease. However, in recent years it was realised that new inflammatory cells might be involved such as Th5, Th9, Th17, y\deltaT cells, NKT cells and their associated cytokines IL-5, IL-9, IL-17, IL-25, IL-33, and thymic stromal lymphopoetin (TSLP), the latter three being derived from the epithelium. Although the epithelium was initially considered to function solely as a physical barrier, it is now evident that it plays a central role in the Th-cells sensitisation process. IgE is the central player in the allergic response too. The activity of IgE is associated with a network of proteins, among them especially with its high- and low-affinity Fc-receptors (Fig. 1). All of these particular players are able to drive immune and inflammatory responses resulting in eosinophilic, neutrophilic or combined clinical forms of asthma what was the subject of our previous paper (2). The aim of our present publication will focus on recent view of clinical complexity of the disease and its contemporary treatment.

Asthma bronchiale phenotypes

Asthma bronchiale is not a single disease but rather a complex of multiple, separate syndromes that overlap. Although clinicians have recognised these different phenotypes for many years, they have remained poorly characterised. However, recent understanding of immunopathological processes enabled to distinguish at least four distinct phenotypes: eosinophilic, neutrophilic, paucigranulocytic, and steroid resistant asthma (3,4,5,6)

Address for correspondence:

Vrlik Mojmir, MD, PhD., Martin Immunology Centre, 036 01 Martin, Mudronova Str.N.12, Slovakia. Phone: ++421 43 4304051; e-mail: pzs@medcentrum.sk



Fig. 1 Periscope function of dendritic cells. Dendritic cells (DCs) intercalate their dendrites between epithelial cells what enables them to sample the airway lumen. DCs form tight junctions with epithelial cells through their expression of adhesive molecules and by E-cadherin homotypic interactions.

Eosinophilic asthma. Eosinophilic airway inflammation is highly characteristic for patients with mild asthma. A broad correlation between clinical asthma severity and the degree of airway eosinophilia has been recorded (7). Moreover, the dramatic reduction of eosinophils in sputum and tissue as the results of asthma treatment with corticosteroids, associated with clinical improvement, has led to the idea that eosinophils are fundamental to airway dysfunction in asthma. However, the role of eosinophils has been recently questioned as the administration of anti-IL-5 monoclonal antibodies, mepolizumab, that reduce their number in the blood and in the sputum, does not reduce airway hyper-responsiveness or asthma symptoms (8). Moreover, anti-IL-5 treatment had no effect on bronchial mucosal staining of eosinophil major basic protein, suggesting that reduction in eosinophil numbers in the blood does not reflect tissue deposition of granule proteins. Therefore, tissue eosinophils may be unresponsive to IL-5 but may instead respond to IL-3 and GM-CSF (9,10). Anyway, eosinophils may be responsible for subepithelial fibrosis and their presence in the airways seems to be a good marker of steroid responsiveness. Also eosinophilic airway inflammation appears much more closely related to the risk of severe asthma exacerbation (11).

Neutrophilic asthma. Some patients with asthma have in their sputum instead of eosinophils, as expected, neutrophils. In general, asthma associated with neutrophils tends to be a more aggressive disease with more tissue destruction and airway remodelling (6,12). Intervention with the etanercept, a p75 TNF- α receptor fusion protein (TNF- α is a potent chemotactic factor for neutrophils) has shown a clinical benefit in such patients (13). It suggests that, as the disease becomes more chronic and severe, inflammatory phenotype changes from Th2 more towards Th17 type as Th17 cells by their IL-17 production induce the release of CXCL8 (IL-8), a neutrophilic chemokine, from airway epithelial cells (14). However, two large randomised control trials using etanercept or golimumab, a fully human monoclonal antibody against TNF- α , failed to confirm this (15); therefore, more investigations are needed to resolve the problem.

Neutrophilic asthma has also been associated with infections. Since asthmatic airway epithelial cells are known to lack ability to mount a primary interferon response following infection with common respiratory viruses (16), it is possible that defective innate immunity may be fundamental to the origins and progression of chronic disease.

A neutrophilic pattern of inflammation is also found in asthmatic patients who smoke. It was shown that alpha-glycoprotein, isolated from tobacco, substantially increased the number of mature DCs in the airways and alveolar walls (17) and that a cigarette smoke had induced high levels of CXCL8. Tobacco smoking is not only associated with a greater neutrophil component, however also, and more importantly, with corticosteroid refractoriness (18). A possible explanation of this is the effect of smoking and oxidative stress in reducing histone-deacetylase activity (see later) in the nuclear chromatin, thereby diminishing the opportunity for corticosteroids to access antiinflammatory genes (19).

Increased oxidative stress by itself is related to disease severity, particularly in a severe disease and during exacerbations. One of the mechanisms whereby oxidative stress may be detrimental in asthma is through the reaction of superoxide anions with nitric oxide (NO) to form the reactive radical peroxynitrite, which may then modify several target proteins. NO is produced by several cells in the airway by NO synthases (20). Current data indicate that the level of NO in the exhaled air of patients with asthma is higher than the level of NO in the exhaled air of normal subjects. The elevated levels of NO in asthma are more likely reflective of inflammatory mechanisms than of its direct pathogenetic role. However, measurement of exhaled NO in asthma is increasingly used as a non-invasive way of monitoring the inflammatory process (21).

Paucigranulocytic asthma. Asthma in the absence of either neutrophils or eosinophils and normal levels of proteolytic enzymes in the patients sputum is termed paucigranulocytic. Proteolytic enzymes play an important role in tissue remodelling and repair, however, their levels and activity varies according to the inflammatory cell phenotype. Subjects with eosinophilic asthma have significantly more active metalloproteinase-9 (MMP-9) compared with those with neutrophilic asthma and control subjects. In neutrophilic asthma, more subjects have neutrophil elastase (NE) activity compared with healthy control subjects, subjects with eosinophilic asthma or subjects with paucigranulocytic asthma (22). Proteolytic enzyme activity in asthma is thus dependent on the underlying inflammatory phenotype and is differentially regulated with MMP-9 activity, a feature of eosinophilic inflammation, and active NE in neutrophilic inflammation. Normal levels of MMP-9 and NE in paucigranulocytic asthma suggests that an abnormal epithelium or underlying mesenchyme and/or smooth muscle may itself lead to an asthma phenotype without the presence of obvious inflammation (4).

Steroid resistant asthma. Glucocorticoids stay in the first-line of anti-inflammatory treatment for asthma. However, a proportion of asthmatic patients fail to benefit from oral glucocorticoid therapy; they are denoted as having glucocorticoid-resistant (steroid-resistant) asthma. The molecular and cellular basis of steroid resistance remains uncertain. Some investigations have shown that steroid insensitivity in these patients is associated with a breakdown of nuclear translocation of the glucocorticoid receptor (23). Some patients who have clinically severe asthma, despite taking oral and high-dose inhaled steroids, show persistent airways neutrophilia and increased expression of both TNF- mRNA and protein. While this suggests a possible role for TNF- in severe asthma, clinical trials of TNF- antagonists have not yet confirmed whether this is a critical element in steroid-resistant asthma (24). It was also shown that CD4⁺ T cells from steroid-resistant asthma patients failed to induce IL-10 synthesis following *in vitro* stimulation in the presence of dexamethasone as compared with their glucocorticoid-sensitive counterparts suggesting a link between induction of IL-10 synthesis and clinical efficacy of glucocorticoids (25).

Pharmacotherapy of asthma bronchiale

Inhaled **corticosteroids** and short- and long-acting β 2-adrenoceptor agonists are now the mainstay of asthma treatment (4,12,26). Corticosteroids suppress inflammation by inducing the recruitment of the nuclear enzyme histone-deacetylase 2 (HDAC2) to multiple activated inflammatory genes, which leads to deacetylation of the hyperacetylated genes, thereby suppressing inflammation. The poor response to corticosteroid treatment seen in patients

with severe asthma, in asthmatics who smoke and during acute exacerbations may also reflect a reduction in HDAC2 enzyme levels and its function. Inhaled steroids also reduce the number of DCs in the lungs and activate indolamine 2,3-dioxygenase in plasmacytoid DCs, thereby broadly suppressing pro-inflammatory responses (13,27). Corticosteroids can damp down airways inflammation, however, they have little effect on the remodelling. This explains why corticosteroids do not abolish all symptoms (4,12,26).

 H_1 -antihistamine treatment for asthma has evolved through a number of phases over the years. Initial enthusiasm for their use in this disorder faded quickly and, for many decades, first-generation H_1 -antihistamines were considered to be contraindicated in asthma due to their anticholinergic effects and potential drying and inspissation of airway secretions. Second-generation H_1 -antihistamines are not harmful in asthma. Although clinical studies have yielded mixed results with regard to efficacy outcomes, in individuals with seasonal allergic rhinitis and concomitant mild seasonal asthma, some second-generation H_1 antihistamines improve rhinitis symptoms and have a modest effect on asthma symptoms. In contrast, **leukotriene antagonists** (LTRA) are clinically effective, confirming that leukotrienes are relevant mediators of asthma. They induce bronchodilation and inhibit airway constriction induced by antigen inhalation or exercise and also exert an anti-inflammatory effect (28). Furthermore, because leukotrienes are known to play an important role in an airway remodelling, LTRA can be used as its inhibitors (29).

Cyclosporine A was found to be of little benefit to asthmatics and is now not recommended as a therapy, particularly because of its toxicity. Tacrolimus, rapamycin and mycophenolate mofetil, which are currently used in the prevention of transplantation rejections, have not been tested in clinical studies of asthma (30).

FTY720, a sphingosine 1 phosphate receptor antagonist, is an immunosuppressant that retains lymphocytes in lymph nodes and the spleen, thus preventing lymphocyte migration to inflammatory sites (31). It has currently been used in clinical trials for the treatment of *multiple sclerosis* and transplant rejection. The accompanying lymphopenia could be a serious side effect that would preclude the use of FTY720 as an anti-asthmatic drug. However, it was shown that the administration of FTY720 by inhalation prior to or during ongoing allergen challenge suppressed the Th2-dependent eosinophilic airway inflammation and bronchial hyper-responsiveness in mice without causing lymphopenia and T cells retention in the lymph nodes. Effectiveness of local treatment was achieved by inhibition of the migration of lung DCs to the mediastinal lymph nodes, which in turn inhibited the formation of allergen-specific Th2 cells (32). However, more studies are needed till the drug enters the clinical practice.

Theophylline has been used to treat asthmatic bronchoconstriction. It is a cAMP phosphodiesterase inhibitor as well as an adenosine-receptor antagonist. Theophylline is reported to have anti-inflammatory effect through increasing activation of HDAC, which is subsequently recruited by corticosteroids to suppress inflammatory genes (33,34). Its cardiac and central-nervous-system side-effects that occur at doses similar to those required to generate a therapeutic effect have led to a marked reduction in its use (12).

Cromolyn sodium has been used in the treatment of allergic diseases, including seasonal and perennial allergic rhinitis, allergic conjunctivitis, vernal keratoconjunctivitis, food allergy and systemic mastocytosis. It was used for the treatment of allergic asthma too, however, the studies have shown that its efficacy is limited what made it to be removed from the WHO list of approved drugs for asthma (35). Also other medicaments have been used in the treatment of *astma bronchiale* (for review see 35,36,37), however, their description is out of the scope of this article.

More specific immunomodulators that selectively inhibit Th2 lymphocytes have been sought for the treatment of asthma. **Suplatast tosilate** administration to patients with bronchial asthma inhibited Th2 cells and Th2-type cytokine release and led to polarisation of circulating Th1/Th2 balance towards Th1 subset. Japan is the only country in the world where the drug is clinically prescribed (38,39).

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Immunotherapy of asthma bronchiale

The sentinel role of IgE in increasing allergen uptake by DCs and activating mast cells and basophils for mediators led to the development of anti-IgE monoclonal antibodies. Nowadays humanised IgG1 antibodies, **omalizumab**, specific for the C_{H3} domain of IgE, are available. They block IgE binding to Fc RI and Fc RII, respectively. Clinical trials have shown that omalizumab administered subcutaneously 2–4 times per week (in dependence of the total level of IgE in the patient's plasma and to the patient's body weight) improves symptom control and allows patients to be treated with lower doses of inhaled corticosteroids. Omalizumab is also effective for the treatment of allergic rhinoconjunctivitis, but therapy has to begin long before the pollen season (12,40).

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Omalizumab effectively neutralises IgE. It is, however, unlikely to affect the long-lived plasma cells that express little of the membrane form of IgE. While the tendency of allergic individuals to mount Th2-cell responses in their target organs persists, there is also the likelihood of relapse if the treatment with the antibody is withdrawn (41).

Low affinity IgE receptor (CD23) plays an important role in the regulation of IgE synthesis. Inhibition of its activity could be thus a promising candidate therapy option for a future treatment of allergic diseases. Really, CD23-specific antibodies, known as **lumiliximab**, were developed. They have already shown their efficacy in a Phase I clinical trial for the treatment of asthma (42).

Because of the principal role of Th2 cytokines in orchestrating allergic inflammation, they and their receptors can be natural therapeutic targets too. IL-4 and IL-13 are crucially involved in the development of allergic responses. Their biological activities start to be realised when bound to their particular receptors. IL-4 receptor (IL-4R) is a heterodimer consisting of its own alpha chain (IL-4R α) and the common γ -chain (γ_c). IL-13 receptor is a heterodimer too; it shares IL-4R α chain in combination with its specific chain (IL-13 α 1). IL-4 signals through both types of receptors, i.e. IL-4R – γ_c , and IL-4R –IL-13R α 1.

A soluble, recombinant IL-4R α protein (**altrakincept**), humanised IL-4-specific monoclonal antibodies (**pascolizumab**), and an IL-4 variant (**pitrakinra**) that targets allergic Th2 inflammation by potently inhibiting the binding of IL-4 and IL-13 to IL-4 alpha receptor complexes have been developed. Unfortunately, all of them failed or had only a weak effect in the treatment of asthma thereby raising doubts over the usefulness of IL-4 blockade for treating of already established allergic disease (43,44,45,46).

Humanised anti-IL-5 monoclonal antibodies (**mepolizumab**) have also been developed. Similar to anti-IL-4 monoclonal antibodies, they had no effect on any measures of asthma outcome in the treatment of patients with severe persistent asthma (8,12). A novel defucosylated anti IL-5R α monoclonal antibodies have been developed (MEDI-563). Engineering of mAbs by removing fucose residues from the Fc fragment leads to greatly enhanced antigendependent cellular cytotoxicity (ADCC) activity as compared to a highly fucosylated conventional antibody. Data from a completed Phase 1 study of MEDI-563 have demonstrated that antibody is well tolerated with substantial and prolonged depletion of blood eosinophils. Perhaps its use will definitively answer the question about the role of eosinophils per se in this disease (35).

Based on the finding that levels of TNF- α are increased in the airways and in blood mononuclear cells in severe asthma, a soluble p75 TNF- α receptor fusion protein, **etanercept**, has been efficiently used for the treatment (47,48). Large multi-centre trials with etanercept and TNF- α specific monoclonal antibodies are now in progress (12).

Prostaglandin D2, the ligand for the G protein-coupled receptors DP1 and CRTH2, has been implicated in the pathogenesis of the allergic response. It was shown that the administration of a highly potent and specific antagonist of CRTH2 to a mouse model of airway inflammation reduced antigen-specific IgE, IgG1, and IgG2a antibody levels as well as decreased mucus deposition and leucocyte infiltration in the large airways (49). These findings suggest a possible new way in the treatment of asthma patients. Similarly, there are reports on anti-IL-9 monoclonal antibodies treatment (50). IL-9 was proved to be produced by

Target	Function	Drug
$\beta 2$ adrenergic receptor	Ultra-long bronchodilation	Indacaterol, carmoterol
Glucocorticoid receptor	Anti-inflammatory	
CRTh2 inhibitors	Th2 cell recruitment and activation	Ramatroban
CCL11	Blocks eosinophil recruitment /activation	
CCR3	Blocks eosinophil recruitment /activation	
IgE	Blocks IgE binding to FceRI and FceRII	Omalizumab
Interleukin 5	Blocks eosinophil recruitment /activation	Mepolizumab
Interleukin 10	Endogenous anti-inflammatory agent	
Interleukin 13	Key driver of asthmatic inflammation	Pitrakinra
JNK	Anti-inflammatory	
CD23	Reduces IgE	Lumiliximab
Sphingosine-1 phosphate receptor	Prevents dendritic cell activity	FTY720
VDR	Increased interleukin-10 expression in Treg cells	Vitamin D3

Table 1. Some of the compounds for asthma treatment (modified from Adcock et al. 2008)

a novel CD4⁺-subpopulation of T cells (51) and IL-9 plays a significant role in driving allergic inflammation. Also other cytokines are considered to be targets of immunotherapeutic interventions (Table 1).

Subcutaneous allergen-specific immunotherapy (SCIT) involves the regular subcutaneous injection of alergen extracts or recombinant allergens using incremental regimens. After repeated exposure to allergen(s) SCIT decreases the recruitment of mast cells, basophils and eosinophils in the skin, nose, eye and bronchial mucosa. SCIT produces an increase in the level of allergen-specific IgA and IgG4 antibodies, and a decrease in the level of allergen-specific IgE antibodies. The induction of tolerance takes from several days to several months. However, once tolerance is induced it can last for several years without further treatment (52).

The limiting factor in SCIT is anaphylactic side-effects, which vary in incidence from 0.1– 5% of individuals depending on severity (53). Improved efficacy with decreased side-effects is the aim of the new approaches to SCIT, including chemically modified allergens (allergoids) (54). Also attaching CpG oligonucleotide motifs to purified allergens seems to be a particularly promising approach to SCIT by increasing the efficacy and decreasing the side effects, as recently reported for the novel ragweed-allergen conjugate (55).

SCIT has been recommended for the treatment of allergic rhinitis, venom hypersensitivity, and mild asthma with only a single or a few allergens involved (12,13). A Cochrane review [www.ginasthma.org., 2008] that examined 75 randomised controlled trials of specific immunotherapy compared to placebo has confirmed efficiency of this therapy in asthma in reducing symptom scores and medication requirements, and improving allergen-specific and non-specific airway hyper-responsiveness. However, in view of the relatively modest effect of allergen-specific immunotherapy compared to other treatment options, these benefits must be weighed against the risk of adverse effects (an anaphylaxis induction).

Sublingual immunotherapy (SLIT) is the administration of allergens to the oral mucosa. Although much higher doses of allergen are required than those used for SCIT, the side-effects are rare and mild what makes this therapy a very suitable, especially for children. Several clinical trials show that SLIT is effective for the treatment of allergic rhinitis caused by grass, olive, ragweed, and birch pollens, as well as rhinitis that is associated with house dust mite and cat dander allergies (12,56).

SCIT and SLIT also decrease the development of sensitisation to new allergens and decrease the risk of new asthma in both adults and children with rhinitis. Several studies have indicated that allergic rhinitis often precedes asthma and therefore it is an important risk factor for the development of asthma (12). We can say that both SCIT and SLIT play an important role in the therapy of allergic rhinitis and asthma but they have to be a part of complex approach to the patient, including anti-inflammatory and other symptomatic medications.

CONCLUDING REMARKS

Bronchial asthma was once considered a purely allergic disorder dominated by Th2 cells, IgE, mast cells, eosinophils, macrophages, and cytokines. However, it is now clear that the disease also involves local epithelial, mesenchymal, and vascular events that are involved in directing allergic reactions to the lung which eventually result in remodelling of the bronchial wall. Understanding of the immunopathogenesis of the disease resulted in a subdivision of clinical forms of the disease into four different phenotypes, eosinophilic, neutrophilic, paucigranulocytic, and steroid-resistant, respectively. Moreover, it has introduced new therapeutic approaches, although corticosteroids are still in the mainstay of asthma treatment. However, we still treat the symptoms and do not cure the disease; to achieve this goal, more understanding of genetics, environmental factors, and immunopathogenesis are needed.

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EXAMINATION OF COUGH AND NON-COUGH SOUNDS BY SPECTRAL AND COMPLEXITY ANALYSIS IN PATIENS SUFFERING FROM RESPIRATORY DISEASES

¹MARTINEK J., ²BENCOVÁ A., ¹TATAR M., ¹VRABEC M., ¹ZATKO T., ³JAVORKA M.

¹Department of Pathological Physiology, Jessenius Faculty of Medicine, Comenius University,

²Clinic of Tuberculosis and Respiratory Diseases, Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic

³Department of Physiology, Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic

Abstract

The aim of our study was to determine, if our recently developed algorithm for distinction between voluntary cough sound and speech in healthy subjects is appropriate for objective monitoring of cough frequency in patients suffering from respiratory diseases. We have recorded 9 patients suffering from lung disease hospitalised at the Clinic of Tuberculosis and Respiratory Diseases, Jessenius Faculty of Medicine, Comenius University in Martin. The recording time was 5 hours (10:00 – 15:00). During the recording time the patients performed their normal daily activities.

From the obtained sound files the characteristic parameters were calculated by time-domain, spectral and nonlinear analysis. The obtained sound files were classified according to these parameters using a classification tree into cough and non-cough sounds. The performance of our developed algorithm was tested by calculating the value of Pearson correlation coefficient. The value of this coefficient did not reach statistical significance (r=0.64; p=0.064).

According to our results we can conclude that our recently developed algorithm for distinction between voluntary cough sound and speech in healthy probands is not suitable for an objective monitoring of cough count in patients suffering from lung diseases.

Key words: cough sound, spectral analysis, non-linear analysis, classification tree

INTRODUCTION

Cough is a normal protective reflex, which clears the respiratory tract and prevents the entrance of noxious materials into the respiratory system (1). Cough is not frequent in healthy subjects, but it is the commonest symptom of many respiratory diseases, forcing the patients to seek a medical advice (1,2,3). To date assessment of cough has been based on subjective evaluation of the patient's perception of this symptom and recording of cough events mostly under non-ambulatory conditions (4). Ambulatory cough monitoring systems which were proposed recently were based either on sound recordings alone (5,6) or on simultaneous sound and electromyography recordings (7,8), but their use has remained restricted to the research settings mainly due to the need of a trained operator to manually identify cough events from the obtained recordings, which is an arduous task.

In order to make cough monitor applicable to clinical practice, it is necessary to develop accurate automatic monitoring system for recording, detection and counting of coughs (9).

Currently, no standardised method exists, and there is no adequately validated generic cough monitor that is commercially available and clinically acceptable. An objective measurement of cough would be of use in clinical practice, clinical research and the assessment of novel therapies. It would permit validation of the presence of cough, grading of severity and monitoring of responses to therapeutic trials (10). Recently we have published an math-

Address for correspondence:

Jozef Martinek, Ing., Department of Pathophysiology, Jessenius Faculty of Medicine, Comenius University, 037 53 Martin, Sklabinska Str.N.26, Slovakia.

Phone: +421 43 423 82 13, e-mail: martinek@jfmed.uniba.sk

ematical algorithm for distinction between voluntary cough sound and speech in healthy probands using spectral and complexity analysis (11).

The aim of the present study was to determine if our recently published algorithm for distinction between voluntary cough sounds and speech in healthy volunteers is appropriate for objective monitoring of cough frequency in patients suffering from respiratory diseases.

MATERIALS AND METHODS

The study was approved by a local Ethics Committee and was performed in accordance with the Helsinki Declaration of 1975 for Human Research.

Subjects

Our study group consisted of 9 patients suffering from lung diseases (bronchogenic carcinoma – 3, acute bronchitis – 2, chronic obstructive pulmonary disease (COPD) – 2, bronchopneumonia – 1 and lymphocytic pneumonia - 1). This group included 3 females - median age 54 yrs, range 52 – 74 yrs; and 6 males - median age 59.5 yrs, range 52 – 78 yrs. All investigated patients were hospitalized at the Clinic of Tuberculosis and Respiratory Diseases, Jessenius Faculty of Medicine and Faculty Hospital in Martin.

Recording system

The algorithm for recording, determination of sounds events and their analysis was described in our previous study (11). Briefly, the sound events were recorded by a portable digital voice recorder (Sony ICD-MX20, Sony Corporation, China) with the sampling frequency of 8 kHz and a miniature omnidirectional condenser microphone (ATR35s, Audio-Technica U.S., Philippines) with a flat frequency response between 50 – 18 000 Hz. The microphone was attached to the subject's chest and was covered by plastic foam membrane to suppress sounds coming from the outer environment. The audio signal from the microphone was initially recorded to the memory card of the digital recorder as a MSV file (memory stick voice file). After recording, the obtained sound files were transferred into PC and converted to 11 kHz, 16-bit mono digital wave file (WAV format) using Digital Voice Editor 2.31 software (Sony Corporation).

Protocol

All recordings were performed in the clinical settings. The recording time was 5 hours (10:00 - 15:00). During the recording, the patients performed their normal daily activities. At the beginning the volunteers coughed voluntarily three times to obtain their individual cough sound pattern.

Determination of sound events and their analysis

The first step consisted of the determination of the sound events from the raw recordings and their isolation. For this purpose we used the moving window with the length of 200 samples, which moved over whole audio signal without overlap. For each position of the moving window the value of standard deviation (SD) was calculated and compared with our empirically determined threshold value. The portions of the signal containing sound events reached relatively high degree of standard deviation, which exceeded empirically determined threshold value. The portions of the signal containing no sound events reached only small value of standard deviation related to the inherent noise present in the signal. The portions of the signal, which were below threshold value were excluded from further analysis and the portions of the signal, being above the threshold value were stored as separate files and underwent further analysis (Fig.1).





Fig. 1. The time progress of 3 sec section of the raw sound recording and the time progress of its standard deviation. For each position of the moving window (0.018s) the value of standard deviation was calculated, and compared to empirically determined threshold value. The portions of the signal containing no sound events reached only low value of standard deviation and they were excluded from further analysis. The portions of the signal with above-threshold values of standard deviation underwent further analysis.

The second step was the calculation of the characteristic parameters of the sound events. These parameters were calculated using time-domain, spectral and non-linear analysis. Using the time-domain analysis we quantified the duration of each determined sound event (parameter *length*). Using the Fast Fourier Transform (FFT) we determined the time progress of power spectral density (PSD). The total power (TP) corresponding to the area under the PSD curve was computed as a measure of the time progress of the sound intensity. From the time progress of the TP we quantified its maximal and mean value (parameters TP_{max} and TP_{mean} , the time of occurrence of the first local and global maxima (parameters *time*_{local} and *time*_{globa}). Parameter *ratio* was determined as the ratio of the sum of TPs of all the local maxima divided by the sum of TPs of all local minima in a given sound event. Division of the value of the first local maximum of TP (TP_{loca}) by the time of its occurrence represented parameter *slope*. The values of sample entropy (SampEn) were computed from the 512 samples around the first local and global maximum. SampEn is a measure of irregularity and unpredictability of the signal. It is higher for noisy and complex signals compared to periodic oscillations. SampEn (m,r,N) is a negative logarithm of the conditional probability that two sequences similar for *m* points remain similar at the next point. Algorithm for SampEn computation was published elsewhere [12]. SampEn was calculated for two values of input parameter r (tolerance; r = 0.1 and r = 0.2 times SD of the window). The length of compared sequence (m = 2) and the length of analysed window (N = 512 samples) was fixed. SampEn for local maximum were denoted as $SampEn_{local}(0.1)$ and $SampEn_{local}(0.2)$, SampEn values corresponding to the global maximum were denoted as $SampEn_{global}(0.1)$ and $SampEn_{global}(0.2)$. From the frequency spectrum determined from these 512 samples around the first local and global maximum we determined the values of skewness and kurtosis of



Fig. 2. An example of the sound parameters calculation from the determined sound event (a). These parameters were calculated using time-domain, spectral and non-linear analysis. From the sound event the time progress of total power was determined (b). From the total power curve the value of first local maximum (TP_{local}) and the time of its occurrence $(TIME_{local})$, the value of global maximum (TP_{max}) and the time of its occurrence $(TIME_{clobal})$ and the mean value from all total power values (TP_{mean}) were determined. From the 512 samples corresponding to the local maximum (c) the value of Sample Entropy was determined. From the frequency spectrum (d) corresponding to these 512 samples the values of skewness and kurtosis were determined.

the PSD values distribution (in the frequency band 0-1000 Hz). The values determined from the first local maximum were named $skewness_{local}$ and $kurtosis_{local}$ and the values determined from the global maximum were named $skewness_{global}$ and $kurtosis_{global}$ (Fig.2).

Classification of the identified sound events

Based on the calculated parameters, the identified sound events were classified into cough and non-cough sounds using classification tree. Trees are directed graphs beginning with one node and branching to many subnodes [13]. We used the classification tree, which was successful in our previous study for distinction between cough sounds and speech (11). The top node of the classification tree contained all identified sound events, which were split according to the characteristic parameters to the cough and non-cough sounds. The performance of our developed algorithm was tested against manual cough counts performed with two trained observers, which was regarded as a gold standard (9).

Statistics

For classification of sound events the classification tree was constructed. Pearson correlation coefficient was used as a measure of agreement between two methods for cough count determination. Statistical analysis was performed using statistical package Systat 10, SPSS Inc.

RESULTS

We performed 5 hours lasting recordings in 9 patients suffering from lung diseases. From these recordings we extracted 13 196 sound files, consisting of cough and non-cough sounds. 2 834 cough sounds and 10 362 non-cough sounds were determined by our mathematical algorithm. 2683 cough sounds were counted manually. The number of cough sounds in particular patients is presented in *Table 1*. The value of Pearson correlation coefficient was r = 0.64 (p=0.064).

Table 1. The results of cough vs non-cough sound classification in 9 patients are shown. The performance of our developed algorithm was tested against manual cough counting, which was performed by two trained observers. This classification was regarded as a gold standard for the evaluation of the classification algorithm effectiveness.

Patient number	automatic algorithm (n)	manual counts (n)
1	445	398
2	412	305
3	39	219
4	179	165
5	89	137
6	677	468
7	608	311
8	194	215
9	191	465

DISCUSSION

In the present study we intended to determine if our recently developed algorithm for distinction between voluntary cough sound and speech in healthy volunteers is appropriate for objective monitoring of cough frequency in patients suffering from lung diseases. As it was shown (see table 1), there are relatively high differences between manual and algorithm cough counts, especially in patients no. 3, 6,7 and 9. The insufficient effectiveness of our algorithm can be caused by fact, that the characteristic parameters of pathological cough sounds are markedly different compared to the characteristics of voluntary cough sounds. In addition, patients were usually trying to suppress coughing, which led to so-called abortive cough, and the cough sound parameters of abortive cough sounds could be markedly different compared to parameters of typical cough sounds in the same patient. Some differences in the manual vs algorithm cough counts can be explained by the ten-

dency of the patients to cough in the cough bouts, which were classified by our algorithm as one cough sound.

We can conclude that our recently developed algorithm for distinction between voluntary cough sound and speech in healthy subjects is not sufficient for objective monitoring of the frequency of cough in patients suffering from lung diseases. Another sound analysis techniques are needed to increase the effectiveness of cough sound analysis for objective cough frequency counting in patients with respiratory disorders.

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PROSTATE-SPECIFIC ANTIGEN PROMOTER POLYMORPHISM AND PROSTATE CANCER RISK

¹Sivonová M., ¹Dobrota D., ¹Mataková T., ^{1,2}Dusenka R., ²Kliment J. jr., ²Kliment J.

¹Department of Medical Biochemistry, Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic

² Department of Urology, Jessenius Faculty of Medicine, Comenius University and Faculty Hospital, Martin, Slovak Republic

Abstract

Prostate-specific antigen (PSA) is an androgen-regulated serine protease that is a part of the kallikrein superfamily, produced predominantly by the prostate and primarily by secretory luminal epithelial cells. The action of androgens is regulated by androgen receptor (AR). After binding to androgen, the AR recognizes and binds androgen response elements (AREs) in the promoter regions of androgen - regulated genes, such as the *PSA* gene. A single nucleotide polymorphism in the ARE-I region at the position -158 relative to the transcription start site of the *PSA* gene was identified. It has been hypothesized that the AR binds the two *PSA* alleles (A and G) with differing affinity and hence may differently influence prostate cancer risk. We have investigated the potential functional significance of this polymorphism and its association with prostate cancer susceptibility in 150 prostate cancer patients and 150 healthy men. *PSA* (G-158A) polymorphism was determined by the PCR-restriction fragment length polymorphism analysis using DNA from peripheral blood samples.

We found no association of the *PSA* G-158Å polymorphism on prostate cancer risk, when comparing the *AG* and *AA* genotypes versus the *GG* genotype. Patients with *AA* genotype had significantly higher serum PSA levels than those with *GG* genotype (p < 0.05). In a case analysis according to the Gleason score, patients with *AA* and *AG* genotype had increased risk for higher Gleason score when compared with patients with *GG* genotype.

Our results showed that the *PSA AA* genotype is associated with a higher serum PSA levels and higher Gleason score and could be considered a risk factor for prostate cancer.

Key words: prostate-specific antigen, gene polymorphism, prostate cancer, Slovak population

INTRODUCTION

Prostate cancer is an important cause of morbidity and mortality in men with advanced age and it is a multifaceted disease in which various environmental and genetic factors play important roles in its development and progression. The prostate is an androgen-dependent organ and polymorphic variants in a number of genes involved in androgen metabolism have been implicated in prostate cancer risk (1,2,3).

Since early detection increases survival rate, the prostate-specific antigen (PSA) test and the digital rectal examination should be offered to men annually beginning at age 50 (4, 5). PSA (also known as KLK3) is a member of the tissue kallikrein family, located on chromosome 19q13.4 and produced predominantly by the prostate and primarily by secretory luminal epithelial cells. Transcription of the *PSA* gene is positively regulated by the androgen receptor (AR), and *PSA* has been extensively studied as a model androgen-regulated gene (6). The AR is a steroid hormone receptor that binds as a homodimer to specific DNA sequences, termed androgen-responsive elements (AREs), and a consensus ARE is located at -156 to -170 from the transcriptional start site of the *PSA* gene. The AR can also bind weakly to sites that differ from the strong consensus ARE, and such a weak nonconsensus ARE (termed ARR) has been identified at -365 to -400 (6,7). The *PSA* gene contains multi-

Address for correspondence:

Sivonova Monika, Mgr. PhD., Department of Medical Biochemistry, Jessenius Faculty of Medicine, Comenius University, Malá Hora 4, 036 01 Martin, Slovak Republic, phone: +421 43 4131565, fax: +421 43 4136770, e-mail: sivonova@jfmed.uniba.sk

ple functional and non-functional single nucleotide polymorphisms (SNPs) in its promoter, which might be associated with the *PSA* mRNA expression and the serum PSA level in prostate cancer patients (8). A single-nucleotide polymorphism, an adenine to guanine substitution at position -158 (G-158A) in the ARE-I sequence of the *PSA* gene was proposed to interact differently with AR, thereby modifying the expression pattern and occurrence of prostate cancer (9).

Given the potential importance to the *PSA* G-158A polymorphism, a number of studies have investigated the association between this SNP and prostate cancer susceptibility in different populations. However, the published data about the association of *PSA* G-158A polymorphism and susceptibility to prostate cancer are controversial. Some studies suggest that this *PSA* polymorphism is associated with prostate cancer susceptibility (9-11), whereas other studies report no association (12,13).

The aim of this study was to study the associations between the *PSA* gene polymorphisms at the position -158 and the risk of prostate cancer, and the relation between the *PSA* polymorphisms and the serum PSA levels in prostate cancer patients.

MATERIAL AND METHODS

Case description

The present case-control study comprised of 150 patients with histologically verified prostate cancer and 150 healthy, unrelated subjects living in the north-western part of Slovakia, who were invited to attend the Department of Urology for regular prostate cancer screening between May 2005 and June 2007. The indication for prostate biopsy was either suspicious finding on digital rectal examination and/or an elevated serum PSA level according to age-specific reference values (40 to 49 years, less than 2.5 ng/mL; 50 to 59 years, less than 3.5 ng/mL; 60 to 69 years, less than 4.5 ng/mL; 70 > years, less than 6.5 ng/mL). Serum PSA levels were performed by the Faculty Hospital Clinical Laboratory using chemiluminescence assay. The Gleason score was determined by histological examination and was available in 116 cases. Healthy, unrelated subjects were all tested for serum total PSA levels, and those with elevated serum PSA level according to age-specific reference values total PSA levels were excluded from the normal controls. Both patients and controls were interviewed regarding age, previous and/or current prostate diseases, incidence of cancer and chronic diseases. The studied population is described in Table 1. The present study was performed under the approval of the Ethical Boards of Jessenius School of Medicine, Comenius University and informed written consent was obtained from all individuals prior to their inclusion in the study.

		Cases	Controls	p-value
No.		150	150	
Age (years)	Mean±SD	61.1±8.3	62.5±10.1	> 0.05
	Median (IQR)	59 (50-78)	60 (50-85)	
PSA (ng/ml)	Mean±SD	23.9±61.7	1.6±1.4	0.0006
	Median (IQR)	7.1 (0.1-150.9)	1.3 (0.1-3.87)	
Gleason score	Mean±SD	6.9±1.5		
	Median (IQR)	7 (4-10)		

Table 1. Principal characteristics of the control and prostate cancer patient groups



Fig. 1 Cleavage of 298 bp PCR products of PSA gene by the NheI restriction endonuclease. Ethidium bromidestained electrophoresed representative PCR-RFLP products samples: 100 bp ladder (lane L), AA allele (lanes 6, 11); AG allele (lanes 1, 2, 3, 5, 7, 8, 9, 10, 12) and GG allele (lanes 4, 13)



PCR-restriction fragment length polymorphism analysis

DNA was extracted from blood samples collected from all subjects by the standard method with proteinase K digestion followed by phenol/chloroform extraction. The *PSA* G-158A polymorphism was determined by a PCR-restriction fragment length polymorphism (PCR-RFLP) assay described by Xue et al (2000) (14). Genomic DNA (100 ng) was amplified in a total volume of 25 1 reaction mixture containing 25 pmol of each PSA primers (forward 5'-TTG TAT GAA GAA TCG GGG ATC GT-3' and reverse 5'-TCC CCC AGG AGC CCT ATA AAA-3'); 200 mol/1 deoxynucleoside triphosphates; 1 U of Taq polymerase in 10 x PCR buffer composed of 16.6 mmol/1 (NH₄)₂SO₄ and 20.0 mmol/1 MgCl₂, pH 8.8. The cycling conditions were 94°C for 10 min, followed by 35 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 40 s with a final cycle at 72°C for 10 min. Each PCR product (10 µl) was digested for 16 hours with restriction enzyme *NheI* (2 U) and electrophoresed on ethidium-bromide-stained 2% agarose gel. The three possible genotypes were defined by three distinct banding patterns: *AA* (298 bp), *AG* (150 and 298 bp), and *GG* (150 bp) (Fig. 1). Genotypes were verified by repeating PCR-RFLP on 40 random samples.

Statistical analysis

The differences between age, serum PSA levels in prostate cancer patients and control group were analysed by Student's t-test. The data are reported as mean \pm standard error of the mean. The chi-square test was used to test the frequencies of genotypes/allele in prostate cancer patients with the control population. The odds ratio (OR), an estimate of the relative risk, with 95% confidence intervals (CI) was computed to assess the relationship of the genotypes/allele to the risk of prostate cancer. All p values cited were two-sided and p values < 0.05 were judged as statistically significant.

RESULTS

Frequencies for the *PSA* G-158A polymorphism in the study groups are summarized in Table 2 and both cases and controls were in Hardy-Weinberg equilibrium. Analyzing the difference in the frequency of *PSA* genotypes between cases and controls, the *AA* genotype had impact on the risk for developing of prostate cancer (OR = 1.4, 95% CI 0.75 - 2.63; p = 0.37).

Mean serum PSA levels, measured at time of diagnosis, averaged 23.9 ng/ml in prostate cancer patients and 1.6 ng/ml in controls (p < 0.001). Table 3 shows an association of *PSA* polymorphism with mean serum PSA levels in prostate cancer patients. The serum PSA levels were significantly higher in the *PSA AA* group than in the *GG* group (p < 0.05). The data correlated with the distribution of high Gleason score (PSA *GG*, 32.2%; *AG*, 33.5%; and *AA*, 34.3%).

Genotypes	Controls No. (%)	Cases No. (%)	OR (95% CI)	p-value
No.	150	150		
GG	42 (28.0)	37 (24.7)	1.00	
AG	74 (49.3)	71 (47.3)	1.08 (0.63-1.88)	0.87
AA	34 (22.7)	42 (28.0)	1.40 (0.75-2.63)	0.37
AA+AG	108 (72.0)	113 (75.3)	1.18 (0.71-1.98)	0.60

Table 2 Distribution of the PSA genotype in controls and patients with prostate cancer

 Table 3 PSA serum levels in the prostate cancer patients in relation to PSA genotype

Genotypes		PSA (ng/ml)	p-value
GG (No. 37)	Mean±SD	8.03±11.7	
AG (No. 71)	Mean±SD	16.37±28.9	0.06
AA (No. 42)	Mean±SD	23.3±46.6	0.03

Table 4. ORs and 95% CIs of PSA polymorphism (AA and AG vs. GG) for Gleason score

Genotype	Gleason < 7 No. (%)	Gleason ≥ 7 No. (%)	OR (95% CI)	p-value
No.	44	72		
GG	14 (31.8)	14 (19.4)	1.00	
AG	23 (52.3)	37 (51.4)	1.61 (0.65-3.97)	0.42
AA	7 (15.9)	21 (29.2)	3.00 (0.96-9.30)	0.09
AG+A/A	30 (68.2)	58 (80.6)	1.93 (0.81-4.57)	0.19

The association of the *PSA* genotypes with Gleason score in 116 prostate cancer patients was analysed (Table 4). Of the 116 patients, 44 (38.0%) had a Gleason score of 4 to 6, and 72 men (62.0%) had a Gleason score of 7 to 10. Relative to genotype *GG*, the OR for high grade (Gleason score \geq 7) versus low grade (Gleason score < 7) disease was increased to 1.61 (95% CI 0.65 - 3.97; p = 0.45) for genotype *AG*, and for the *AA* genotype to 3.00 (95% CI 0.96 - 9.30; p = 0.09).

DISCUSSION

In this study we evaluated the allelic frequencies of the *PSA* polymorphisms at position - 158 in Slovak men. It has been shown that the prevalence rate of the *GG* homozygosity is more common in Japanese-Americans (64%), compared with Hispanic (37%), non-Hispanic white (29%) and black American (24%) populations and European white populations (30%)

(14-16). The GG genotype was 28% in our control subjects. This frequency is also similar to the frequencies found in other studies that analyzed PSA G-158A polymorphism in Caucasian population (10, 16).

Previous studies have suggested that the PSA G-158A polymorphism is associated with the risk of prostate cancer or its disease progression but the results concerning the association of the A or G allele and the prostate cancer risk differed among the studies (9,10, 17, 18). It has been hypothesized that AR binds the two allelic variants AGAACAnnnAGTACT and AGAACAnnnAGTGCT with different affinities, leading to differences in PSA expression (9). Lai et al (2007) have used a number of sensitive *in vitro* assays to identify a possible functional role for the G-158A polymorphism in AREI of the PSA gene, by showing that the A allele confers greater androgen responsiveness and shows a greater affinity for the AR than the G allele (16). They have also demonstrated that men in an Australian Caucasian population with an AA genotype had a 3-fold increased risk for developing prostate cancer and men with an AG genotype had a 2.4-fold increased risk. Our results also suggest that AA genotype of PSA gene could modulate the risk of prostate cancer; even if this association did not reach statistical significance and our results are compatible to other studies (19, 20). The variation in published prostate cancer prevalence rates can be attributed partly to methodological differences in survey design; some of these studies have been conducted in small sized groups, in populations of different ethnicities, and in studies with varying numbers of cases and controls. The easiest way to improve precision is to increase the number of subjects and patients in the experimental design. However, this may not be applicable to all research conditions due to such factors as additional costs, poorer availability of resources, lower population, which compromises the number of subjects eligible for the investigation.

PSA serum concentration has long been used as a tumor marker for monitoring prostate cancer progression. Several case-control studies have investigated the relationship between *PSA* polymorphism and serum PSA levels with contradictory findings (9, 15, 20). Higher serum PSA levels have been shown to be associated with the *A* allele or *AA* genotype (9, 15). The *GG* genotype has also been demonstrated to be associated with higher serum PSA levels (20, 21). Our data are in agreement with the findings of Xue et al (2001), patients with the homozygous polymorphic *PSA* genotype (*AA*) have a significantly higher serum PSA levels than those with the *GG* genotype (9).

The cases were analysed according to the Gleason score. We have found that *PSA AG* and *AA* polymorphisms were more common in patients with a Gleason score of 7 or greater (80.6%) than in those with a Gleason score of less than 7 (68.2%). In contrast Gsur et al (2002) have found that the *GG* genotype was significantly more frequent in patients with Gleason score \geq 7 than in patients with Gleason score < 7, providing evidence that the *G* allele is associated with more advanced disease at the time of diagnosis (10). Otherwise, they have found that men having at least one *PSA G* allele were at statistically significant decreased risk of developing a prostate cancer. They hypothesized of an ambivalent role of PSA during prostate carcinogenesis, that is, it has both stimulatory effects on prostatic cell proliferation (22) as well as anti-angiogenic properties (23).

In conclusion, our study demonstrates that *PSA* G-158A gene polymorphism may play an important role in the risk of prostate cancer, mainly in men with the higher serum PSA levels and *PSA* polymorphism might be one of the molecular marker for prostate cancer. We assumed that disease risk could be also influenced by other gene variants, either within the *PSA* gene itself or in other genes, such as the *AR* gene.

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AGGRESSIVE-GROWTH TYPES OF BASAL CELL CARCINOMA OF THE SKIN

¹ BARTOS V., ²ADAMICOVA K., ³PEC M.

¹Department of Pathological Anatomy, Faculty Hospital, Zilina

²Institute of Pathological Anatomy, Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic ³Institute of Medical Biology, Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic

Abstract

Basal cell carcinoma (BCC) of the skin is recently the most common form of cancer in human population with continuously increasing incidence. In contrast to the other malignancies a typical feature of BCCs is their clinical indolence characterised by slow growth, low invasivity, minimal metastatic potential, and high cure rate. Therefore, a disease has usually a favorable prognosis and a complete surgical excision is almost always curative. However, certain types of BCCs demonstrates a more aggressive biological behaviour accompanied by a destructive growth, deep local tissue infiltration, and resistance to standard treatment with frequent relapses. Actually, these rarer forms of tumors are classified as agressive-growth types of BCCs and histologically include infiltrating sclerosing and non-sclerosing variants, micronodular and metatypical BCC. At the light microscopy, especially the infiltrating variants commonly manifest as a poorly-demarcated lesion composed of irregularly sized and shaped nests of neoplastic cells showing widespread invasion of the dermis and penetration into the subcutaneous tissue. Becasuse of not sharply circumscribed contours, a complete tumour excision is more difficult to perform and consequent recurrences after treatment are frequent. Although various factors have been postulated as parameters of the aggressive BCC phenotype, most likely not neoplastic cells alone, but especially a complex stromal reaction is responsible for aggressive biological behaviour of BCCs and their resistence to local therapy. Many reciprocal interactions among neoplastic cells, stroma, and inflammatory tissue response play an important role in pathogenesis of disease. There is need to search continuously reliable prognostic indicators that can predict outcome in patients with aggressive-growth types of BCCs and may detect individuals at high risk for recourrence and metastases. Majority of the clinically important predictive factors are derived from a pathologist's examination of tissue specimens. Recently, molecular analysis with identification of genes that might play a role in acquired aggressiveness of certain BCCs might provide some insights into the pathogenesis of this disease and represent the focus of our future studies targeting to novel therapeutic strategies.

Key words: basal cell carcinoma, biological behaviour, prognostic factors

INTRODUCTION

Basal cell carcinoma (BCC, basalioma) constitutes approximately 70-80 % of all malignant skin tumours and currently is the most common malignancy in human population (1, 2, 1)3). Morover, its incidence is steadily increasing. Microscopically, BCC is composed of proliferating keratinocytes which arises from the basal layer of the epidermis or the pilosebaceous adnexa (1, 4). In fact, this neoplasia represents a relatively diverse subsets of tumours with various clinical manifestations in accordance with the presence of various morphological features, which to a certain extent correspond with the histological types. In contrast to the other human malignancies a characteristic feature of BCCs is their clinical indolence. BCC generally has a relatively innocuous course characterized by slow growth, only minimal invasiveness, and a high cure rate (1, 4). In most cases, a progression of disease is slow, a metastatic potential is very low and metastases occure in extremely rare cases (1, 2). That is why the disease has typically a favorable prognosis, as complete surgical excision is almost always curative (4). However, some types of BCCs demonstrates more aggressive biological behaviour characterised by a destructive growth, deep local invasion ("rodent ulcer"), and resistance to standard treatment. Thus, BCCs significantly contribute to the overall morbidity of the general population

Address for correspondence:

Bartos Vladimir, MD., 036 01 Martin. Str. P.Mudrona N.30/16;

Mobile: 0908/386 352; e-mail: vladimirbartos@post.sk, bartos@jfmed.uniba.sk

Histological diagnostics and classification of BCCs are essential for the determination of the tumour type and its biological behaviour. These informations are very important for the clinician specialist to set up a futher treatment plan and dispensing of the patient (5). The only proven histological prognosticator of biological behaviour, and therefore a major determinant of what constitutes an appropriate therapeutic approach, is the architectural growth pattern. Thus, the microarchitecture is a critical issue, while the differentiation patterns need to be considered only insofar as they must be recognized as part of the histological spectrum of BCC, and as they impact differential diagnosis (1). Although there is no unique and generally accepted classification of BCCs, according to biological behaviour of the individual types, these tumours can be practically categorized to 2 main subgroups : a) aggressive (high risk), and b) indolent (low risk) carcinomas (1, 3, 6).

Indolent (non-aggressive) types include especially nodular and superficial BCC, in which the treatment and prognosis are usually excellent. Aggressive types represent the infiltrative-growth forms, micronodular, and metatypic (basosquamous) BCC. Clinical prognosis of these types is much worse, not only because of possible subclinical spread and higher reccurrence rate, but also because they can metastasise even before developing or having any of the other risk factors. Therefore, a prompt treatment and follow-up is recommended.

HISTOLOGICAL VARIANTS OF AGGRESSIVE TYPES OF BCCs

Infiltrating variants of BCCs

The infiltrative-growth forms represent a special subgroup of BCCs and histologically include infiltrative non-sclerosing and sclerosing (morpheaform) variant (5). The both variants constitute approximatelly 10 % of all types of BCCs (5). At the light microscopic level, infiltrative non-sclerosing forms of BCCs comprise irregularly sized and shaped nests of tumor cells showing widespread invasion of the reticular dermis and penetration into the subcutaneous tissue (Fig 1). These nests usually show sharp angulation of their peripheral contours, increased mitotic activity and occasionally individual cells necrosis (1, 5, 7). Roughly one-third of such cases show an admixed nodular component from which the lesions are held to derive following UV irradiation (8). Sclerosing/morpheaform variant is characterised by columns of basaloid cells enmeshed in a densely collagenized stroma containing large amount of fibroblasts. Grossly, this results in keloid scar appearence of the lesions (5, 7, 9). According to histomorphological similarity (although only distant) with a localised form of scleroderma called "morphea", it is routinely named as morpheaform BCC in the clinical



Fig 1. Infiltrative non-sclerosing BCC comprise irregularly sized and shaped nests of neoplastic cells showing wide-spread invasion in the dermis; (H&E 40x, sample obtained from the archives of the Department of Pathology in Zilina).

practice. The original term invasive basalioma proposed by Trapl and Bednar had continuously disapeared from the nomenclature (5, 10, 11, 12). This is rare variant representing roughly 1-5 % of all BCCs (1, 13) and having some special clinical differences in contrast to the other types of BCCs. Morpheaform BCCs occur most frequently on the face around nose, eyes, and temporal areas (11, 12, 13). It has usually an appearence of a poorly-demarcated and slightly depressed, elastic white-yellowish skin lesion. Like a non-sclerosing variant (1), it has just inconspicious marginal contours and usually a smooth surface without ulcers or crusts (1, 13). Therefore, it seems rather like a scar than a true skin neoplasia. Becasuse of a poorly circumscribed contours, an actual margins of the lesions are often more extensive than is clinically apparent. This is the reason why a complete tumor excision is more difficult to perform and consequently recurrences after treatment are more common. The most critical are the anatomic sites of the embryonal tissue fusions, as nasolabial fold, medial cantus, or retroauricular region (7, 9, 13). In such cases, Mohs micrographic surgery is the treatment of choice providing the most precise resection of the tumor margins (7, 13). Moreover, the superficial layers of the morpheaform BCCs often have a solid growth pattern and the infiltrative type is present in the lower or peripheral layers of the tumor. If excision is not complete, a tumor can be classified as a nodular type and the infiltrative type is detected only during re-excision (5).

Micronodular BCC

Micronodular BCC macroscopically presents as elevated or flat plague-like indurated lesion with a poorly demarcated contour. Histologically, it manifests neoplastic nests with roughly the same shape and contour as nodular BCC, buth which are nonetheless smaller and widely dispersed in an often asymmetric distribution expending deeper into the dermis or subcutis (Fig 2). The surrounding stroma shows either a myxoid or collagenized morphology. The lesions may be difficult to remove, the surgical margins may be underestimated, thus it has an increased incidence of reccurrence (1, 14).

Basosquamous carcinoma

The basosquamous (metatypical) BCC is characterised by infiltrating jagged tongues of neoplastic cells, some of which manifest an abortive peripheral palisade and basaloid morphology, admixed with other areas showing cytoplasmic keratinisation and intercellular bridge formation (Fig 3). Some examples of this variant may merge with sebaceous carcinoma as lipid vacuoles or ducts may be focally apparent. Crowson (1) proposes to distinguish this neoplasm from keratotic BCC, which is most often a nodular BCC with squamous differen-



Fig 2. Micronodular BCC demonstrates small neoplastic nodules expanding in the dermis. Note border between tumour and stroma is very poor; (H&E 100 x, sample obtained from the archives of the Department of Pathology in Zilina)

Fig 3. Metatypical BCC is characterised by infiltrating aggregates of neoplastic cells, some of which manifest an abortive basaloid morphology (thin arrow), admixed with other areas showing cytoplasmic keratinisation (thick arrow); (H&E 100x, sample obtained from the archives of the Department of Pathology in Zilina).



tiation, and from the mixed basal cell – squamous cell carcinomas, that represent a collision between two clonally distinctive and geographically separate neoplasms. Metatypical BCC has no distinguishing gross clinical features, but it has a more aggressive behaviour and has been associated with regional and widespread metastases (1, 15).

HISTOMORPHOLOGICAL FACTORS RELATED TO THE AGGRESSIVE BIOLOGICAL BEHAVIOUR

Although etiopathogenesis of BCCs is generally well-known, particularly involving a chronic ultraviolet sun-light exposition of the skin, immunosupression and a genetic susceptibility in some cases, our knowledge about mechanisms responsible for aggressiveness and invasivity of certain subtypes of this carcinoma is rather vague. It is very likely, that not carcinoma cells alone, but especially a stromal reaction is responsible for aggressive biological behaviour of BCCs and their resistence to local therapy. Like in carcinogenesis of other malignant tumors, many reciprocal interactions among neoplastic cells, stroma, and inflammatory tissue response play an important role in pathogenesis of this disease. Modification of tumour-associated connective tissue indicates a close relationship between the carcinoma cells and the adjacent matrix in order to product tumour-growth permissive tissue. Characteristic stroma alterations accompany or even precede the malignant conversion of epithelial cells.

First of all, an alteration of basement membrane integrity is of great importance in the pathogenesis of infiltrative-growth variants of BCCs. Ten years ago Hayakawa et al. (16) proved, that both the superficial and nodular variants of BCCs are surrounded by a continuous basement membrane zone, but the aggressive-growth variants (ie morpheaform, meta-typical and infiltrating subtypes) manifest an absent basement membrane. It has been suggested (1), that an activation of the matrix metalloproteinases results in digestion of basement membrane material in process of neoplastic transformation. Thus, this perhaps iniciates a progression of the indolent forms of carcinomas to more aggressive-growth variants. Besides the exact histological tumour classification, it is necessary for pathologist to reliable predict a possibility of the following biological behaviour not just in morpheaform, but all types of BCCs (11, 12). This particularly includes a description of tumour periphery, and a detection of basement membrane integrity. Adamicova et al. (11, 12) consider to be histochemical Gömöri staining method the most sensitive for detection of basement membrane

integrity defects what, in fact, allows a prediction of a probability of the following biological tumor behaviour.

Until now, there have been studied many biological factors and various molecular marksignifficantly affected the tumour-stroma interaction and tumour progression. It is ers known that aggressive-growth variants of BCCs are associated with a nuclear p53 overexpresion and stromal hyperplasia (8). P53 seems to correlate well with a "dedifferentiation process" in BCCs (17) and tumours that overexpress p53 have a poorer prognosis (18). Indolent-growth forms of BCCs are in majority accompanied by an overproduction of antiapoptotic Bcl-2 protein (19), whereas its low level correlates with clinically aggressive-variants of BCCs (6). From the perspective of further prognosis and treatment, detection of these both markers in routine biopsy specimen can bring a significant predictive information about a disease. For instance Zagrodnik et al. (20) observed that patients treated with radiotherapy had shown the most frequent recourrences in sclerosing type of BCC, which have immunohistochemically manifested a significant higher p53 expression and low expression of Bcl-2. Staibano et al (21) proposed, that the finding of clones expressing Bcl-2 may be indicative of an indolent phenotype of BCCs on head and neck regions, and thus Bcl-2 could be used as a "clonal marker" of a favourable clinical behaviour. The partial or complete loss of Bcl-2 during histologic transformation, with the appearance of clones expressing p53 protein in a BCC could be considered a hallmark of transition from a lowgrade to a high-grade malignancy.

In the process of neoplastic cell transformation and progression to more invasive forms, the alteration of actin-myosin complex may also be one of the crucial factors. Some authors (22, 23) have demonstrated higher level of immunohistochemical -smooth muscle actin expression in both, tumorous and even benign stromal cells in more aggressive forms of BCCc. It is almost obvious that increased protein contractility in human neoplastic cells results in their higher motility, and thus it increases their invasivity and metastatic potential. Christian et al. (23) showed actin expression in 0 % of nodular, 66 % of micronodular, and in 62 % of morpheaform BCCs. In another study (24) actin occurred in 25 % of nodular and 100% of infiltrative BCCs. Law et al. (22) compared a presence of cytoplasmic actin in simple nodular BCC (N-BCC) versus mixed nodular-infiltrative BCC (NI-BCC) and they had demonstrated actin positivity in 28 % of N-BCCs and in 100 % of infiltrative component of NI-BCCs. Moreover, Bozdogan et al. (6) demonstrated significant differences in reciprocal expression of Bcl-2 protein in the neoplastic cells and -SMA expression in the stroma. They found, that non-aggressive BCCs have shown concomitant staining, while the aggressive BCCs have demonstrated discordant type of immunoreactivity. That means, shortly, increased -SMA expression in benign stroma in combination with decreased or absence Bcl-2 expression in the neoplastic cells might be probably highly suggestive for further aggressive biological behaviour of BCCs. The other biomarker which has a myoepitheliumlike contractile character is calponin. Some authors (25) revealed that it is much more prevalent in mixed nodular-infiltrative or infiltrative BCC variants in contrast to the simple nodular type. They supposed, calponin may be one of the most important factors influencing a metastatic potential of the neoplastic cells. However, on the other hand, Uzquiano et al. (24) recently did not show statistically significant differences in calponin expression among individual subtypes of BCCs. Another important indicator of biological behaviour appears to be a syndecan-1, whose intracellular expression become lost with increasing aggressiveness of BCC (26). However, within the dermis, which is normally devoid of this marker, immunopositivity for syndecan-1 is present in areas adjacent to aggressive tumours. This pattern of staining indicates that syndecan-1 is produced by stromal cells rather than being shed by the carcinoma cells into the stroma (26). It was also demonstrated in one study (27) that a proliferative activity of neoplastic BCC cells is associated with increased expression of hyaluronan in the stroma. Especially in infiltrative-growth BCCs, such alterations include degeneration and possible further remodelling of the surrounding extracellular matrix. That is evidence that a metabolic activity and structure of

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the stroma adjacent to carcinoma are closely related to cytological aspects and proliferative activity of the neoplastic cells.

In a lesser extent, loss of adhesion molecules on the surface of the epithelial cells also plays an important role during tumour progression. Normally functioning cell-cell adhesion plays an crucial role in the maintentance of tissue architecture and cohesion. E-cadherin is one of the most important adhesion molecule in epithelial cells. Pizzaro et al. (28) observed a significantly reduced E-cadherin expression in specimen of infiltrative BCCs in contrast to superficial and nodular types. These results suggest that E-cadherin might be related to the growth pattern and the local aggressive behaviour of BCC. Tumour growth may be also limited by the expression of integrins by binding to the surrounding stroma, but no significant differences in the amount or pattern of expression was seen in the different histologic subtypes of BCCs (29). In study of Saldanha et al (30) nuclear beta-cate-nin expression in BCCs correlated with increased cellular proliferation so it may also have a potential biological effect in tumour progression.

It is well-known that any tumour growth beyond a certain size requires angiogenesis. Evaluation of the formation of new blood vessels has been proposed to provide important prognostic information also in BCCs. The quantification of new microvessels within a tumour can be commonly performed using antibodies against factor VIII or CD 34 antigen to identify endothelial cells. Staibano et al. (31) demonstrated, that all samples of aggressive types of BCCs showed a significantly higher microvessel count than non-aggressive types. This suggests that the angiogenic process may be an important step in the acquisition of the aggressive phenotype in human BCCs and there is a correlation between tumour vascularization and clinico-biological parameters of aggressiveness in BCC. Microvessel density evaluation can be beneficial in selection of those individuals, which have BCC with the highest risk of reccurrence or metastatis.

The other important factor which influences the aggressiveness of BCC is a perineural invasion (PNI). Although it is an uncommon histological feature of BCC, when present, it is associated with more aggressive forms of tumours. Infiltrating, morpheic, and basosquamous types are more likely to be associated with PNI (32). Brown et al (33) found an incidence of perineural invasion of 3 % in the aggressive BCC types. The preauricular and check regions appear to be the most common sites of BCC with PNI, involvement of the facial and trigeminal nerves usually occure first.

DISEASE PROGRESSION AND METASTASIS

As was mentioned above, various factors, including microarchitecture and growth pattern have been postulated as histological parameters of the aggressive BCC phenotype. Furthermore, in recent years, numerous molecular markers have been studied to explain its pathogenesis and relatively indolent behaviour. However, the underlying molecular mechanisms of tumour invasion and metastasis of BCC have yet to be elucidated. The development and progression of BCC is accompanied by mechanisms, the nature of which is still incompletely understood. Several studies have shown that ultraviolet radiation is responsible for the induction of p53 mutations and thus for the initiation probably of both indolent and aggressive forms of BCCs (34, 35). However, the fact that both forms contained the same ultraviolet signature p53 mutations suggests that this genetic alteration may play a role in tumor initiation but not in tumor progression (34). It is undetermined whether individual intrinsic biological factors within certain subsets of BCC predispose these tumors toward an inherently aggressive behavior, or whether any BCC with inadequate early management may assume this phenotype (4). It is interesting and worth noting, that the indolent-growt variants are topografically found widely distributed on both sun-exposed and sun-protected skin, while the aggressive growth-variants are much more frequent in sunexposed skin (19). Based on this fact it has been proposed (4, 19), that lesions occurring on

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those different areas of the body can have also a fundamentally different biological characteristics.

Kaur et al. (36) lately postulated the following theoretical histological stepwise model of BCC progression : superficial \rightarrow nodular \rightarrow micronodular, or superficial \rightarrow nodular \rightarrow infiltrative \rightarrow morpheaform. They confirmed a significant linear correlation with host response and alteration of tumor stroma in their study (36). Based on this, we can assume that various BCCs exhibit distinct epithelial-stromal-inflammatory patterns that correlate with BCC subtypes, tumour progression and neoplastic evolution from "low risk" to "high risk" form of BCCs. It has been proposed (14) that in such histological spectrum of BCCs, the intermediate step between the indolent and aggressive-growth subtypes would probably be a micronodular BCC. However, it has been also speculated (9), whether all infiltrating types of BCCs necessarily always evolve from another indolent BCCs, or they can be present ab initio, too. Assuming that the morpheaform BCCs are derived from commoner types of BCCs, thus it would be rather a secondary phenomenon of tumor progression than original pathology. On the other hand, prior scarring or long-standing irritation of the skin could at least in some cases directly initiate the tumorous changes to precisely this type of carcinoma. Monitoring of development sequence of BCC is very difficult or almost imposible in clinical practice, because the overwhelming majority of tumours is removed after a certain period of growth during which histomorphology and biological character of carcinoma could be changed.

If we accepted theory of "multistep model" of BCC progression or not, there is still no consensus to what extent the infiltrative-growth variants of BCCs further predisposes to metastatic disease (37, 38). Metastases occur in extremely rare cases with an incidence of only 0,0028–0,5 % of all BCCs (37). Although metastatic BCC cases have generally showed a higher incidence in the more aggressive histologic patterns (39), this issue is still debatable. It is true, that among 12 reported cases by Lo et al (40), 11 patients had the morpheaform histological variant of BCC, however, all the lesions were either recurrent, large, or invading deeper structure. Snow et al (41) revealed the overall rate of metastases for morpheaform BCC less than 1 %, which is very small percentage of the incidence. Generally, given the very unique occurrence of metastases there is a comparision of biological characteristics of primary and secondary BCC practically impossible. Therefore, the factors determining which BCC will metastasize have not been clearly elucidated. However, many authors have not demonstrated pronounced morphological changes in metastatic BCCs until now. For example, several studies have examined the role of various cellular adhesion molecules in tumor metastasis, but no correlation was noted in their expression or distribution by different histologic subtypes of BCC (29, 42). In the study of Baum et al (42), an integrin profile of BCC did not differ essentially from that of metastasizing tumour varieties and can not be regarded as a major reason for the non-metastasizing phenotype of BCCs. Similarly, acccording to Giri et al (43), in acquiring metastatic potential, tumors did not lose the molecular characteristics of Bcl-2 and CD 44 expression (cell surface marker), the two features deemed to be important in the indolent behavior of BCC. Uzquiano et al. (24) noted, that although increased amount of actin in neoplastic cells undoubtedly influences the local invasivity of BCCs, it does not markedly occur in metastatic tumours. There were also no obvious differences observed by electron microscopy between metastatic and non-metastatic BCCs (44). It should be noted, however, that it is difficult to draw definitive conclusions about the relationship between invasive potential and the differences in the expression of cellular markers because of the phenotypical heterogenity between individual tumour subtypes.

Today, several specific categories of genes are identified in individual forms of BCCs which may determine the particular clinical and histological BCC phenotypes. Genetic analysis indicated that gene expression patterns of BCC subtypes in multiple biological processes showed significant variation, particularly in genes associated with the mitogen-activated protein kinase pathway (45). Yu et al (45) recently found, that specific genes have typical-

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ly presented with greatest fold change in expression in morpheaform BCCs in contrast tu nodular and superficial BCCs and genetic alterations involved in response to DNA-damage stimulus were uniquely upregulated in morpheaform BCCs. Their results indicate a relative similarity in gene expression in indolent forms of BCCs, but more diverse in morpheaform BCCs in which gene expression patterns is consistent with their invasive phenotype.

CONCLUSION

There is a need to search continuously reliable prognostic indicators that can predict outcome in patients with aggressive-growth types of BCCs and may detect individuals at high risk for recourrence and metastasis. Majority of the clinically important predictive factors are derived from a pathologist's examination of tissue specimens. Recently, molecular analysis with identification of genes that might play a role in acquired aggressiveness of certain BCCs might provide some insights into the pathogenesis of this disease and represent the focus of our future studies targeting to novel therapeutic strategies.

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TREATMENT OF SCAPHOID FRACTURES AND PSEUDOARTHROSIS IN CHILDHOOD

¹STRANSKA M, ¹MUDRAK I., ²KRAJCOVIC A., ¹DRIMAL J.

¹Clinic of Ortopedics and Traumatology, Jessenius Faculty of Medicine, Comenius University, and Faculty Hospital, Martin;

²Department of Public Health, Jessenius Faculty of Medicine, Comenius University, Martin, Slovak Republic

Abstract

Scaphoid fractures in childhood, i.e. before the completion of ossification, occur rarely. This fact is usually the cause of both misdiagnosis and wrong treatment. During diagnosis in childhood patients a fracture or distal epiphyseolysis of radius are taken into consideration rather than a possibility of scaphoid fracture itself. The aim of our work is to present our experience in treatment of scaphoid injuries in children and, at the same time, to point at a relatively high percentage of unrecognized fresh fractures of scaphoid.

Between 2003 and 2007 there were 12 childhood patients treated for a fracture and pseudoarthrosis of scaphoid at our Clinic. Four of these patients were treated conservatively as a result of fresh fracture diagnosis, in two patients a percutaneous Herbert screw osteosynthesis for inveterate scaphoid fracture was performed, and six patients were operated on for scaphoid pseudoartrosis.

After evaluating the results of treatment in patients using the Mayo score (15) there was an excellent outcome in 5 patients treated for scaphoid fracture and in 4 patients operated on for scaphoid pseudoarthrosis. Good results were observed in 1 patient after fresh fracture treatment and in 1 patient after treatment of scaphoid pseudoarthrosis. Bad results were observed in 1 patient after treatment of pseudoarthrosis during which the screw became loose and dislocation of fragments occurred and a reoperation was necessary.

Key words - fracture, scaphoid, pseudoarthrosis, Herbert screw, childhood

INTRODUCTION

Scaphoid fractures are very rare in persons under the age of 15 (1). According to Cook they make up less than 1 per cent of all fractures (3), according to Műssbichler (18) they make up 0.45 per cent of all upper limb fractures, according to Christodoulou and Colton (13) they make up 2.9 per cent of all fractures of wrist and hand, and Mintzer states that they make up 93 per cent of all carpal bone fractures (17).

Scaphoid ossification begins in children between the age of 5 and 6 and it is completed between the age of 13 and 15 (21). Before the scaphoid ossification is completed the scaphoid is almost entirely made up of cartilage (19). A thick layer of cartilage surrounding the ossification nucleus provides an increased flexibility of scaphoid and reduces vulnerability to a fracture (5,8). Thus, a greater force is needed for a fracture of ossificating scaphoid due to its elasticity compared to force needed for a scaphoid fracture in adults (9).

The mechanism of injury which will probably result in Colles fracture in elderly patients will probably cause scaphoid fracture in adolescents and young adults and it will probably induce distal radial epiphyseolysis in children aged 5 - 11 and/or subperiostal fracture in toddlers (10).

According to Christodoulou and Colton the scaphoid fracture in patients under the age of 15 is in most cases located at the distal pole and the fracture is most often incomplete or nondislocated (13).

According to Boles (2) an increased spread of high-energy sports has resulted in the same location of scaphoid fractures in both adults and children. Seventy per cent of scaphoid fractures are localised in the middle third of scaphoid (in the narrowest point of scaphoid termed as "waist" in Anglo-Saxon literature), 10 to 20 per cent of the fractures are localised

Address for correspondence:

Stranska Maria, MD., Clinic of Ortopedics and Traumatology, Jessenius Faculty of Medicine and Faculty Hospital, Kollarova Str. N.2, 036 59 Martin, Slovak Republic

in the distal third of scaphoid, 5 to 10 per cent of the fractures are localised in the proximal pole and 5 per cent in tubercle.

METHODS

In a retrospective study we analyzed 12 patients aged 9 to 15 over 5 years (2003 to 2007). They were treated for a fracture and pseudoartrosis of scaphoid at our Clinic. In all 12 patients we used an X-ray examination in four projections, the so-called Scaphoidal Quartet, for diagnosis and in patients with scaphoid pseudoarthrosis this examination was supplemented with CT examination. Based on these examinations and using Herbert classification we determined the type of the fracture or pseudoarthrosis and decided on its treatment.

In all patients with fresh nondislocated scaphoid fractures (see above) we decided on conservative treatment. We loaded a circular plaster fixation from the IP joint of the thumb and heads of the 2nd to 5th metacarps up to the elbow. The limb was immobilized untill the Xray examintion revealed that the fracture had been healed. After removal of the plaster fixation we recommended rehabilitation treatment considering the invidual functional medical finding.

In patients where the scaphoid fracture was not primarily recognized and treated and where the fracture was inveterate we used percutaneous Herbert screw osteosynthesis for the treatment. Patients were operated on under general anesthesia or in intravenous Bier block. Surgical approach was chosen depending on the location of the fracture.

With fractures localised in the distal third of scaphoid we used a palmar approach. We percutaneously inserted Kirschner wire from the distal end of scaphoid to its proximal end under X-ray control. The small incision was made at the place of the wire insertion. Then we pre-drilled the scaphoid using the inserted Kirschner wire as a guiding for the hollow drill. Finally, the Herbert screw was inserted to the scaphoid and the Kirschner wire was removed. Following the suture of the wound an elastic bandage was loaded and after removal of the suture the patients started with rehabilitation. Full load of the limb was permitted after the X-ray verified that the the fracture had been healed.

In patients with scaphoid fracture in proximal and middle third we used a dorsal approach. Insertion of the Herbert screw and subsequent immobilization were the same as in the previous procedure.

In patients with scaphoid pseudoarthrosis we chose an open approach. Following the localisation of pseudoarthrosis on the border between central and distal thirds of scaphoid we decided on palmar approach in all cases. The incision was led proximally from the scaphoid tubercle and radially from the tendon of flexor carpi radialis muscle. After incision of the joint capsule we revealed the place of pseudoarthrosis and resected its edges. Afterwards we inserted spongious pelvic graft or graft from the distal radius using a "press fit" technique. Then we inserted the Kirschner wire under the X-ray control, pre-drilled the scaphoid using the wire and fixed the preudoarthrosis with the Herbert screw. After the surgery we loaded a plaster splint for the period of 4 to 6 weeks. In two patients a brace was loaded because the consequent X-ray control revealed an incomplete bone consolidation.

RESULTS

Between 2003 - 2007 there were 12 patients at the age of 9 to 15 treated for fracture or pseudoarthrosis of scaphoid at our Clinic. Four patients were treated conservatively for a fresh fracture (the mean age of the patients was 12.9), two patients with fracture with dislocation of scaphoid fragments were treated with percutaneous Herbert screw osteosynthesis (the mean age of the patients was 14.2) and six patients were operated for scap-

Fig. 1. The Herbert classification of fractures and pseudoarthroses. In Green DP.: Operative hand surgery. 3rd ed. New York, NY: Churchill-Livingstone, 1993.



hoid pseudoarthrosis (the mean age of the patients on the date of the accident was 13.9 and on the day of the surgery the mean age of the patients was 14.6). Out of all scaphoid fractures treated at our Clinic (49 patients with scaphoid fracture) the childhood fractures constituted 12.3 per cent. Pseudoarthroses constituted 21.4 per cent of the total number (28 patients with scaphoid pseudoarthroses). In our group there were 10 boys and 2 girls (a nine-year-old treated conservatively for a scaphoid fracture and a fourteen-year-old treated with percutaneous Herbert screw osteosynthesis for an inveterate and dislocated scaphoid fracture). Seven cases were injuries of the right scaphoid and five cases were injuries of the left scaphoid. In all our patients the cause of their fractures was a fall to extended wrist. The causes of their falls were as follows: sports injuries (3 patiens – hockey 2x, football 1x), traffic accidents (2 patients - fall off a bicycle) and 7 injuries were caused by an ordinary fall. According to the Herbert classification (Fig.1) there was one fracture of the type A1, one fracture of the type A2, one fracture of the type B1, and three fractures of the type B2. There were six pseudoarthroses according to the Herbert classification, three fractures of the type D1 and three fractures of the type D2.

Between 2003 and 2007 there were 4 patients treated conservatively (Table 1). All cases were fresh scaphoid fractures of the types A1 (1x), A2 (1x) and B2 (2x). The patients were 3 boys and 1 girl. Three patients had injury of the right scaphoid and one patient had injury of the left scaphoid. The mean age of the patients on the date of the injury was 12.9. The mean length of plaster immobilization was 4 weeks, all fractures were healed after the period of immobilization, and pseudoarthrosis had not developed in any of these cases. The functional outcome after the treatment in these patients was a painless and unlimited range of motion in the injured wrist.

Two patients were treated by the percutaneous Herbert screw osteosynthesis (Table 2). Both cases were dislocated scaphoid fractures (1x B1 type and 1x B2 type) unrecognized at other departments. The period from the injury to the osteosynthesis was in both cases 1.5 months. The mean age of the patients on the day of the surgery was 14.2. One patient had injury of the left wrist and the other one had injury of the right wrist. The fixation with an elastic bandage lasted in both cases 2 weeks. After the fixation the patients started with



Fig. 2. X-rays of the wrist, anteroposterior and lateral views, showing the fresh scaphoid fracture in a 13 years old boy

Fig. 3. X-rays of the wrist, anteroposterior and lateral views, showing the healed scaphoid fracture in a 13 years old boy

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Table 1. Patients with conservative treatment of scaphoid fractures

2003 - 2007	AGE OF PATIENT	TYPE OF FRACTURE	AFFECTED LIMB	PERIOD OF IMMOBILIZATION
boy	13years 2months	B2	right	5 weeks
boy	14years 5months	A2	right	4 weeks
boy	14years 8months	A1	right	4 weeks
girl	9 years 4months	B2	left	3 weeks

rehabilitation and in 2 months after the surgery they were allowed full load on the wrist. The patients do not complain of pain and they have full range of motion in the wrist. Six patients aged 12 to 15 were operated on for pseudoarthrosis at our Clinic (Tables 3

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Fig. 4. X-rays of the wrist, anteroposterior and lateral views, showing the inveterate scaphoid fracture in a 14 years old boy



Fig. 5. X-rays of the wrist, anteroposterior and lateral views, showing the healed inveterate scaphoid fracture in a 14 years old boy after Herbert screw osteosynthesis



 $\textbf{Table 2.} \ \textbf{Patients treated for inveterate scaphoid fracture}$

2003 - 2007	AGE OF PATIENT ON INJURY DATE	AGE OF PATIENT ON SURGERY DATE	TYPE OF FRACTURE	AFFECTED LIMB	PERIOD OF IMMOBILIZATION	FULL LOAD
Boy	14years 3months	14years 5months	B2	right	2 weeks	8 weeks after surgery
Girl	13years 8months	14years	B1	left	2 weeks	8 weeks after surgery

and 4). Their mean age on the date of injury was 13.9 and 14.6 on the day of their surgery. All patients were boys, in 3 of them the pseudoarthrosis was in the left scaphoid and in 3 patients it was localised in the right scaphoid. In 3 cases the pseudoarthrosis was of the

type D1 and in 3 cases of the type D2. In all patients an open reposition with spongioplastics and Herbert screw fixation was used. For all patients we used the palmar approach with respect to the pseudoarthrosis localisation. With the exception of 1 patient the pseudoarthrosis had been healed in all patients. X-ray revealed the full consolidation of pseudoarthrosis and full load was permitted in five patients within 4 to 6 months (on average 5 months) after the surgery. In one patient a failure of osteosynthesis and dislocation of fragments occurred in 7 months after the surgery and a reoperation was required.



Fig. 6. X-rays of the wrist, anteroposterior and lateral views, showing the scaphoid pseudoarhrosis in a 15 years old boy



Fig. 7. X-rays of the wrist, anteroposterior and lateral views, showing the healed scaphoid pseudoarthrosis in a 15 years old boy after spongioplasty and Herbert screw osteosynthesis

2003 - 2007	AGE OF PATIENT ON INJURY DATE	AGE OF PATIENT ON SURGERY DATE	TYPE OF PSEUDOARTHROSIS	AFFECTED LIMB
Boy	14 years 4 months	15 years	D2	left
Boy	14 years 3 months	14 years 11 months	D1	left
Boy	14 years 2 months	14 years 10 months	D2	right
boy	12 years 9 months	13 years 4 months	D1	right
Boy	13 years 8 months	14 years 4 months	D1	left
Boy	14 years	15 years	D2	right

Table 3. Patients treated for scaphoid pseudoarthrosis

Table 4. Patients treated for scaphoid pseudoarthrosis

2003 - 2007	AGE OF PATIENT ON SURGERY DATE	USED GRAFT	PERIOD OF PLASTER IMMOBILIZATION	PERIOD OF BRACE IMMOBILIZATION	FULL LOAD
Boy	15 years	from hip bone	1 month	3 mounths	4 mounths after surgery
Boy	14years11months	from distal radius	1 month	_	5 mounths after surgery
boy	14years 10 months	from hip bone	6 weeks	_	5 mounths after surgery
boy	13 years 4 months	from distal radius	1 month	-	7 mounths after surgery osteosynthesis failure
boy	14 years 4 m.	percutaneous osteosynthesis	6 weeks	2 mesiace	6 mounths after surgery
boy	15 years	from hip bone	6 weeks	_	5 mounths after surgery

Table 5. The results of scaphoid fractures and pseudoarthroses treatment expressed in Mayo wrist score

Mayo score	FRACTUF	RES PSEUDOARTHRO	SES	
	n	%	n	%
90-100	5	83.3	4	66.6
80-90	1	16.7	1	16.7
60-80	-	-	-	-
< 60	_	_	1	16.7



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Out of 12 patients the fractures were recognized immediately after the accidents in 4 of them and in all four cases the fracture was healed by means of conservative treatment. Six patients were examined immediately after the accident but due to the negative X-ray finding the fracture was not diagnosed and an adequate treatment method was not applied. A plaster splint was loaded for one week only in 3 patients. However, control X-rays were not made after removal of fixation in any of them. Fractures or pseudoarthroses were found in these patients in control X-ray images made several weeks or months afterwards when patients visited the doctor for persistent pain in the wrist. Two patients did not seek medical examination after the accident and they came to see a doctor after longer period of time following the accident because of pain in the wrist. The analysis of our group of patients shows that in 50 per cent of the children the scaphoid fracture was not primarily diagnosed. Since the fracture was not even suspected the patients were not adequately treated and, consequently, they had to undergo surgery.

DISCUSSION

The scaphoid fractures occur in children rarely since the thick layer of cartilage surrounding the nucleus of ossification provides increased flexibility of the scaphoid and reduces vulnerability to a fracture (5, 8). Most fractures in children are without dislocation and can be successfully treated by the limb immobilisation (8, 13, 17, 23).

We have to keep in mind that the scaphoid fracture in children may be overlooked for several reasons. In the X-ray image the thick layer of cartilage may mask the fracture line, the symptoms may be typical for an injury in distal forearm where injuries are more frequent than in carpal bones and the X-ray image in childhood is not unambiguous (12).

Out of six fractures treated at our Clinic four of them were revealed immediately after the accident. These four fractures were treated with the limb immobilisation and in all four patients we achieved excellent results according to the Mayo wrist score after their treatment (Table 5).

Two patients were treated with percutaneous Herbert screw osteosynthesis for inveterate and dislocated fractures, in one patient the result was excellent and in the other we achieved a good result -limited dorsal wrist flexion (Table 5).

Most pseudoartrhroses are localised in the middle third of the scaphoid (so-called waist) (6, 7, 11, 12, 14, 16, 22, 24) as it was in all our patients treated for pseudoarthrosis in our group. The pseudoarthroses in the distal pole of the scaphoid are rare (6, 7, 11, 16, 20, 24). The largest set of pediatric patients with scaphoid pseudoarthroses was published by Chloros (12). His set consisted of a group of 12 patients in which surgery consisting of the following steps was carried out: open reduction, spongioplasty of scaphoid with corticospongious graft taken from the pelvic bone, and retrograde Herbert screw fixation. According to the Mayo score (4) this technique achieved excellent results in 11 patients and good results in 1 patient.

Our group consisted of 6 patients treated for scaphoid pseudoarthroses. In 4 patients we achieved an excellent result according to the Mayo score, in 1 patient the result was good and in 1 patient we recorded a bad result (Table 5).

Based on our experience we may conclude that in all patients in which the fracture was primarily diagnosed correctly the fracture was healed after a four-week immobilization with no need of a surgical treatment. In inveterate dislocated fractures the treatment with percutaneous Herbert screw osteosynthesis proved as a suitable method and for the treatment of pseudoarthroses the most suitable method is transplantation of autologous bone graft with additive spongioplasty with Herbert screw fixation.

We consider early diagnosis of scaphoid fractures as the most important. Whenever the scaphoid fracture is suspected the wrist immobilization and repeated X-ray examinations

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are required to confirm or exclude the fracture. According to personal preferences and accessibility of particular diagnostic examinations we recommend a CT examination of the scaphoid. The CT scans should be carried out in layers along the long axis of the bone and the thickness of the layer should not exceed 1 mm. MRI examination which represents a lower load of radiation for the patient is less available in the acute phase and it is less suitable for displaying the scaphoid morphology. The plaster immobilization of the carpus for 10 to 14 days followed by an X-ray check which were recommended in the past, should be used exceptionally today. It seems that even today when CT and MRI are commonly used the diagnosis of scaphoid fractures is still a diagnostic problem.

CONCLUSION

Scaphoid fractures in childhood are rare injuries. In many cases they are forgotten during the diagnosis of carpus injuries, which leads to increase of complications arising from late determination of correct diagnosis. In the group of patients treated at our Clinic the early unrecognized fractures made up to 66 per cent.

A high percentage of primarily unrecognized scaphoid lesions in children from our group is determined by the concentration of patients who are sent with complications to our Clinic from other workplaces. Finally, we would like to point out that the diagnosis of scaphoid fractures in childhood should not be forgotten and if such diagnosis is suspected, correct methods of diagnosis and treatment shall be used.

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