Cytokeratin 8 and 18 tissue expression in gallbladder mucosa of patients with cholelithiasis

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Cytokeratins (CKs) 8 and 18 are normally expressed in simple epithelia. This unique pair of CKs is believed to be involved in hepatic diseases and many human cancers. Little is known about the role of tissue expression of both CKs in patients with cholelithiasis (CH). The aim of the study was to analyse tissue expression of CK8 and 18 in the specimens of gallbladder mucosa in 35 young (up to 25 years of age) and 20 older patients (approximately 50 years of age) with CH. An immunocytochemical ABC method and the spatial visualization technique were conducted. Our study demonstrated significantly lower amounts of both CKs in young patients, as compared to older patients. The higher cellular expression of CK8 in older patients was linked to acute clinical course vs. chronic ones. Tissue expression of neither CK correlated with inflammatory activity (grading) of the gallbladder mucosa. A positive correlation between reciprocal expressions of the two CKs may confirm a cytoprotective role of the two proteins in both groups of patients with symptomatic CH. Significantly higher expression of CK18 than that of CK8 in younger patients suggests a different role of CK8 and 18 in lithogenesis in this group.

Key words: gallstone disease, cytokeratins 8 and 18, immunocytochemistry, spatial visualization technique.

Introduction

Gallstone disease belongs to diseases linked to civilization and 10-15% of white adults in developed countries harbour gallstones [1]. The role of cholelithiasis (CH) and of metaplastic lesions of gallbladder epithelium in the course of chronic cholecystitis is accentuated [2]. Factors associated with a high risk of CH continue to include female gender, obesity, insulin resistance, genetic conditioning and age [3-5]. Cholelithiasis is manifested more frequently in older individuals although in recent years an increase has been observed in the incidence of symptomatic CH in children and young adults [6, 7]. Manifestation of CH in young patients understandably induces interest since causes of CH most frequently remain unknown and the problem is increasingly urgent. Better techniques are sought for earlier diagnosis, therapy and, first of all, prevention of CH in this age group [6].

Cytokeratins (CKs) represent a very extensive group of polypeptides, the expression of which preferentially takes place in epithelial cells [8]. These proteins are encoded by a large multigene family that includes more than 50 individual members [9]. The proteins are an important component of the intermediate filament system and the physiological role of CKs has been mostly recognised [8-10]. Cytokeratins 8 and 18 are normally expressed by simple epithelia, while

in vitro studies showed that increased CK8 expression changes the phenotypic characteristics of non-transformed human fetal buccal mucosa cells to those of transformed cells [11].

In normal human gallbladder epithelium expression of mainly CK8 and 19 can be noted, and lower quantities of CK7 and 18 [12]. Cytokeratins 18 is recognised to represent a specific marker of brush cells in epithelia of the respiratory tract and alimentary tract (including gallbladder), in which it probably plays a receptor role [13-15]. Ectopic expression of CK20 was noted in some human gallbladder adenocarcinomas [16].

In order to evaluate the role of CKs in lithogenesis, their expression was examined in normal gallbladder epithelium of wild-type mice and in the epithelium of mice with knocked out genes of CK8, 18 and 19. A lithogenic diet induced a more severe liver injury in CK8-null than in wild-type mice without altering keratin protein levels [17]. A tendency to form gallstones proved to be comparable in healthy mice and mice with *CK8* gene knockout. Wild-type gallbladders do overexpress keratins upon lithogenic diet challenge. Lithogenic diet-induced injury triggers keratin hyperphosphorylation in wild-type livers and gallbladders [17].

Due to the poorly recognized role of CK8 and 18 in pathogenesis of CH in humans, in this study we decided to perform an analysis of gallbladder tissue expression of CK8 and 18 as correlated with selected clinical data especially in young patients (up to 25 years of age) with symptomatic CH. For comparison, patients most frequently subjected to cholecystectomy in the same period were studied (of approximately 50 years of age) with the same diagnosis.

Material and methods

Patients

All the patients were subjected to cholecystectomy in the Municipal Hospital in Ostrów Wielkopolski, Poland. Group A (n = 35; young patients) included all patients up to 25 years of age (16 to 25 years of age, 29 women and 6 men) who were diagnosed and subjected to surgery in 2003-2007. For the reference group (group B) the age criterion was accepted, amounting to approximately 50 years since in the years this was the most frequent age of CH patients subjected to surgery at the Ward of Surgery of the above-mentioned Municipal Hospital. Twenty patients were selected for the group (48 to 50 years of age, 15 women and 5 men). Duration of CH symptoms in the analysed groups of patients most frequently ranged between 6 months and one year.

In both groups of patients, gallbladders were fixed in buffered 10% formalin and embedded in paraffin using the routine procedure and were subjected to staining with haematoxylin and eosin (HE). The histopathological diagnoses exhibited traits of chronic cholecystitis in all patients from group A and B. The analysed clinical data included age, gender, results of laboratory tests, data related to anamnesis, including the data on clinical course of the disease directly before surgery, duration of symptoms and body mass index (BMI) upon admission to the hospital. In both groups of patients mostly mixed, cholesterol/pigment/calcium gallstones were detected. However, their detailed chemical composition was not tested. We had no group of control patients with normal gallbladders since in Poland due to potential complications cholecystectomy is not performed on other surgical procedures. Also we had no access to gallstone-less gallbladders, subjected to surgery for urgent indications. For evaluation of CK expression, gallbladders with well-preserved mucosal structure were selected, examined in at least 10 microscope fields at the objective magnification of 40×. Under the term of "acute" symptoms (acute course of diseases) we understood an acute pain, a positive Murphy sign, increased WBC and/or fever. In both groups of patients, histological examination confirmed the earlier established diagnosis of chronic cholecystitis. The number of gallstones disclosed following cholecystectomy was estimated using a semi-quantitative 1-4 point scale [18]. Written informed consent was obtained from each patient before the operation, and approval for the study was granted by the institution's Ethical Committee (No. 281/08).

Tissue material

The studies were conducted on serial, 5 μm paraffin sections, placed on the SuperFrost/Plus microscopic slides. Patterns of HE-stained gallbladder histological preparations were examined using an Olympus BH-2 light microscope by two experienced histopathologists (WB, AK) blinded to the clinical details on the histopathology request form. Thickness (width) of the total wall of gallbladder was measured (in mm) in HE-stained paraffin sections.

Each tissue specimen was also evaluated based on a simple numerical scoring system for the grade of *lamina propria* inflammation (G1) (0-3) and the grade of muscularis externa/adventitia inflammation (G2) (0-3), as described in our earlier studies [18].

Immunocytochemistry and microscopy image analysis

Studies on cellular localisation of CK8 and 18 in gallbladder mucosa took advantage of the classic ABC (strept(avidin)-biotinylated peroxidase complex) method according to Hsu *et al*. [19], the individual stages of which were described in detail in our earlier reports [20]. Mouse anti-human monoclonal antibodies (mAbs) were

employed, directed against cytokeratin 8 (Sigma-Aldrich; clone M20; in 1 : 100 dilution) and CK18 (Sigma-Aldrich; clone CY-90; in 1 : 800 dilution). Then, the sections were treated with primary mAbs at night at 4°C, then secondary biotinylated link anti-mouse and anti-rabbit IgG and with the ABC complex (Dako REAL™Envision™, Dako, Glostrup, Denmark). Following deparaffinization and rehydration the preparations were additionally boiled in 10 mM citrate buffer in a 700 W microwave oven for 18 min, washed in PBS, and then subjected to the reaction according to the standard procedure. Every test was accompanied by a negative control in which specific antibodies were supplemented by a normal serum of a respective species in 0.05 M Tris-HCl, pH ~7.6 supplemented with 0.1% bovine serum albumin (BSA) and 15 mM sodium azide.

Histological slides with CK8 and 18 tissue expression were examined under the optical Olympus BH-2 microscope coupled to a digital camera. Colour microscope images were recorded using 40× magnification objective (at least 10 fields in every microscope slide with an immunocytochemical positive reaction) and LUCIA Image 5_0 computer software (2560 × 1920 pixels in size), documenting them in jpg format on the computer hard disk (total number of preparations with a positive reaction for CK8 was 530 images and for CK18, 490 images).

The quantitative evaluation of the CK8 and 18 tissue expression was performed using the image processing method, including spatial visualization of markers in microscope images, elaborated and programmed in the A4D computer software C++ language by Strzelczyk [21] and described in detail in our previous paper [18]. In this study results related to expression of CK8 and 18 were presented in % of the immunocytochemical reactions manifested by the entire mucosa (Fig. 1).

Results obtained using the A4D spatial visualization software were compared with results of colour thresholding in HSI space obtained using Image J software (public domain, NHI, Bethesda, MD) applied to the same images. In this aim, the Color Thresholder plug, available at http://rsb.info.nih.gov/ij web page, was installed in the basic version of the software. Results ob-

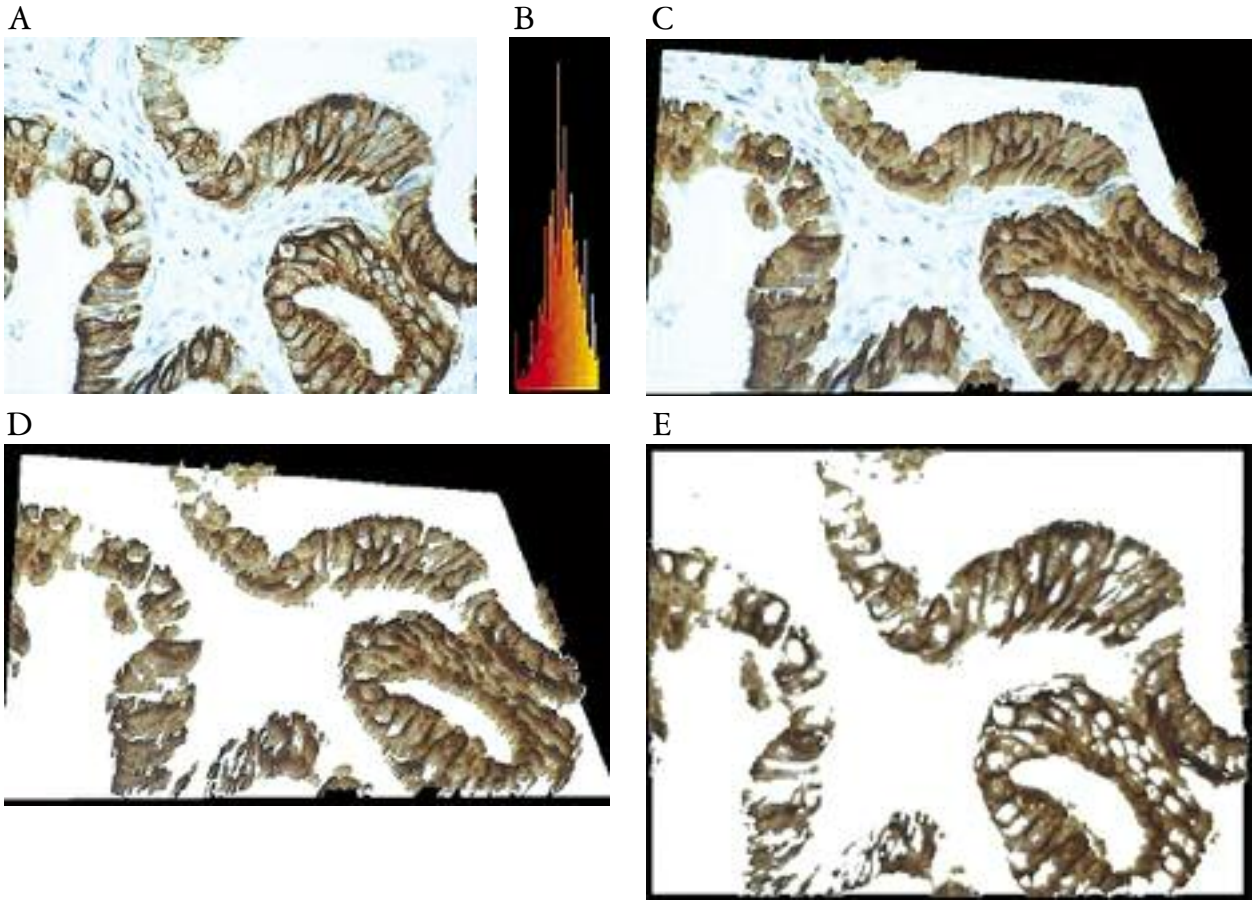

Fig. 1. Working window of A4D software. Threshold segmentation of colour immunocytochemical reaction (brown colour) in the example of cytokeratin 8 (CK8) expression in gallbladder epithelium of a patient with symptomatic cholelithiasis. (A) image obtained under a light microscope coupled to a digital camera. Objective magnification: 40×. ABC technique, cell nuclei counterstained with haematoxylin; (B) histogram of colours composing shades of CK8 brown colour; (C) spatial transformation of a flat microscope image (A); (D) segmentation of CK8 expression from a spatial image (C); (E) projection of the spatially transformed reaction on a plane as a final result of automatic CK8 segmentation for measurement of its area and of its share (%) in area of gallbladder mucosa

tained in the two programs for image analysis (ImageJ and A4D) were exported to the format of Microsoft Excel, which is compatible with Statistica PL v. 8, with the aim of conducting statistical analysis.

Statistical methods

At the first stage of the statistical analysis, consistency of the obtained results was checked with the Gauss distribution, using the Shapiro-Wilk test. Subsequently, parameters of descriptive statistics were calculated (arithmetical mean, standard deviation, median value, minimum and maximum values). Results of spatial visualization technique studies were compared between the young patients (group A) and older patients (group B) (unlinked samples) using the Mann-Whitney test. Spearman's rank correlation was applied to examine the association between variable values. The statistical analysis was performed using the Statistica PL v. 8 software. Differences and relationships were accepted to be statistically significant at the level of $p \leq 0.05$.

Results

Clinical and histopathological data

Body mass index was significantly higher in older patients than in young individuals ($p = 0.004$). Also a significantly higher WBC number was found in older than in young patients with symptomatic CH ($p = 0.008$) (Table I). Inflammatory activity of the gallbladder (G1, G2) in young patients was similar to the 50-year-old patients ($p = 0.504$ and $p = 0.335$, respectively). Selected clinical data for both groups of patients are summarized in Table I.

Table I. Selected clinical data, and results of laboratory tests in young (A) and older (B) patients with symptomatic cholelithiasis (mean ±SD)

PARAMETER	A	B	P
Age (years)	21.60 ±2.50	49.10 ±1.21	0.001
BMI (kg/m^2)	25.04 ±3.84	29.17 ±4.01	0.004
Number of gallstones	2.43 ±1.12	1.95 ±0.83	0.182
Width of gallbladder wall (mm)	5.63 ±2.07	5.51 ±2.02	0.869
WBC ($\times 10^9$/l)	7.83 ±2.78	11.11 ±4.55	0.008
PLT ($\times 10^9$/l)	223.87 ±60.41	238.60 ±65.25	0.213
Bilirubin (mg/dl)	1.51 ±1.69	0.78 ±0.32	0.173

*A – group of patients below 25th year of age; B – group of 50-year-old patients; BMI – body mass index; WBC – white blood cells; PLT – platelets; *inflammatory activity (grading) score see Material and methods; p – level of significance*

Cellular localisation of CK8

Expression of CK8 was detected in all tissue samples (100%) of both examined groups. It involved very numerous cells in gallbladder epithelium (Fig. 2A). Positive expression was detected exclusively in cytoplasm of columnar cells in gallbladder epithelium (Fig. 2B). A more intense positive reaction involved the perinuclear space and lateral surfaces of the neighbouring columnar cells. The positive reaction was present both in the morphologically intact epithelium and in the regenerating one (Fig. 2C). Expression of CK8 was also detected in tubuloacinar glands (Fig. 2D).

Cellular localisation of CK18

Expression of CK18 also was detected in all tissue samples of the patients (100%) in each group and involved most gallbladder epithelial cells (Fig. 3A), probably also including brush cells (Fig. 3B). The most pronounced reaction was detected in the perinuclear space and on lateral surfaces of columnar cells. Expression of CK18 also included cells of regenerating epithelium (Fig. 3C) and of tubuloacinar glands (Fig. 3D).

Quantitative evaluation of CK8 expression in both groups of patients

Significantly lower expression of CK8 in gallbladder mucosa was detected in the group of younger patients as compared to the group of 50-year-old patients (Fig. 4).

Quantitative evaluation of CK18 expression in both groups of patients

Significantly lower expression of CK18 in gallbladder mucosa was also detected in the group of younger patients as compared to the group of older patients (Fig. 4).

Differences and reciprocal relations between CK8 and 18 expression

In the younger patients an almost twofold higher expression of CK18 was demonstrated as compared to expression of CK8 in gallbladder mucosa (Fig. 4). In the older patients expression of CK8 and 18 manifested a similar intensity (Fig. 4). A positive correlation was demonstrated between reciprocal expression of CK8 and 18 in gallbladder mucosa in each group of patients (Fig. 5A and B).

Relationships between CK8 and 18 expression and histopathological/clinical data

No significant Spearman's correlations could be noted between CK8 and/or 18 tissue expression and inflammatory activity of the gallbladder (grading), BMI of the patients, number of gallstones, width of gall-

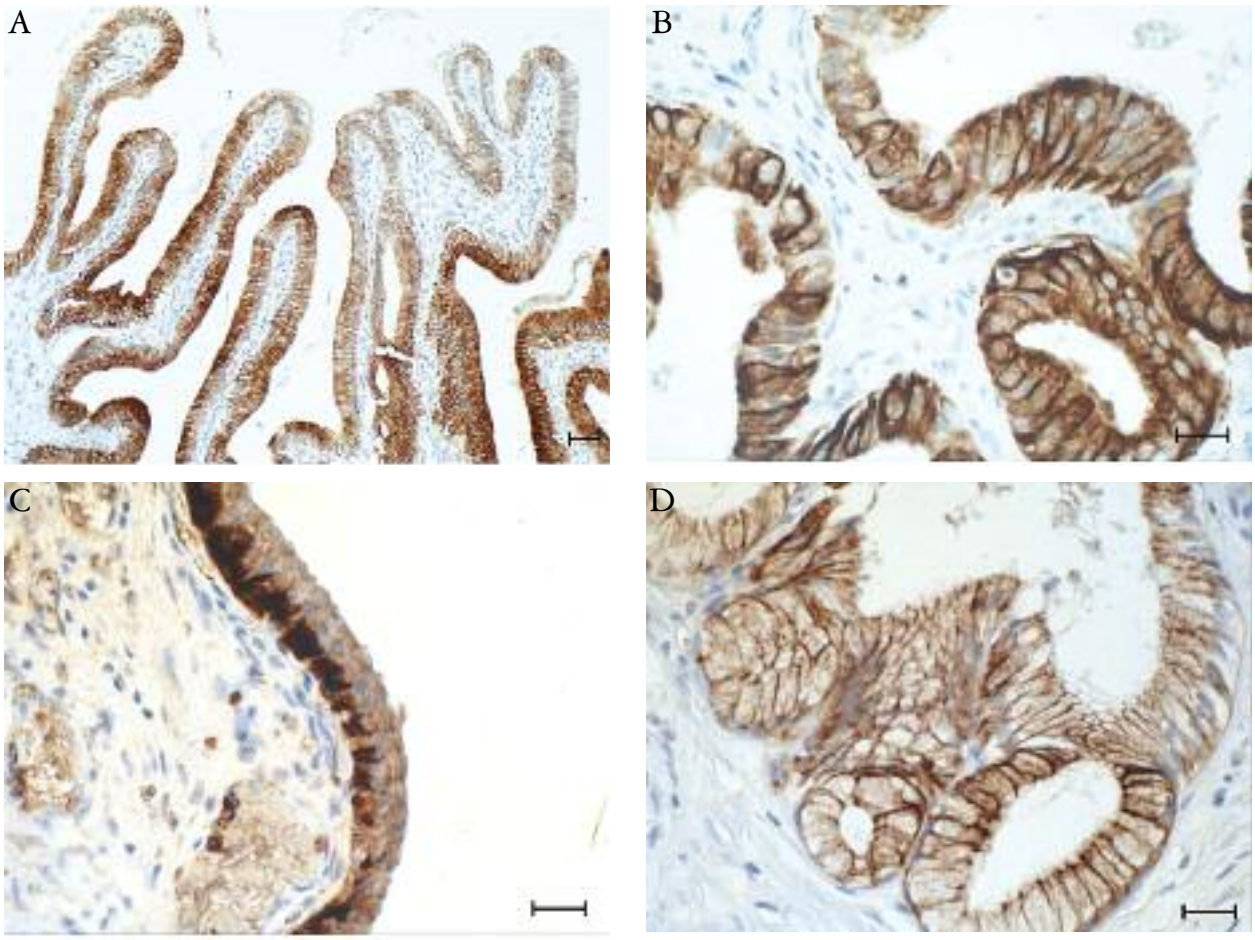

Fig. 2. CK8 expression in gallbladder mucosa of patients with CH: (A) evident positive reaction in cytoplasm of the most epithelial cells; (B) localisation of CK8 mostly on perinuclear and lateral surfaces of columnar cells; (C) CK8 basolateral localisation in regenerating gallbladder epithelium; (D) weak positive reaction of CK8 in tubuloacinar glands in *lamina propria*. ABC technique. Haematoxylin counterstained. Bar = 40 μm (A), 20 μm (B-D)

bladder wall, WBC and platelets number ($p > 0.05$). Only in group B was a negative correlation disclosed between tissue expression of CK18 and serum bilirubin level in the patients (Table II).

Significantly higher expression of CK8 was detected in older patients as compared to younger patients independently of the clinical symptoms upon admission to the hospital (acute and chronic) ($p = 0.002$ and $p = 0.001$ respectively). In group B there was noted significantly higher expression of CK8 in patients with acute clinical symptoms upon admission as compared to those with chronic symptoms. Cytokeratin 18 tissue expression was also higher in older patients in comparison with younger patients but only in those with chronic clinical symptoms (Table III).

Discussion

In this study we investigated tissue expression of CK8 and 18, because few data are available on their role in pathology of gallbladder [17], and the profile of their expression in cultured cells of gallbladder is poorly defined [22], while reports on their involvement in hepatic diseases [23, 24] encourage extension of the studies to gallbladders with gallstones, particularly in young patients. In normal human gallbladder epithelium expression of mainly CK8 and 19 can be noted by other authors, and lower quantities of CK7 and 18 [12]. The proteins are an important component of the intermediate filament system and play important mechanical and non-mechanical functions [8-10]. Keratin hyperphosphorylation correlates with exposure to a variety of stresses in cultured cells and in mouse models of liver, pancreatic, and gallbladder injury, and it is found in association with mouse and human Mallory bodies [25].

Some of the studies have stressed cytoplasmic expression of the so-called apoptotic fragments of CK18 in epithelial cells of gallbladder in all patients with chronic cholecystitis [26]. Total concentrations of CK18 and the so-called M30 neo-epitope were higher in bile of the patients as compared to their serum. On the other hand, no significant differences were noted in detectability of the above forms of CK18 which would be related to activity of the inflammatory process [26].

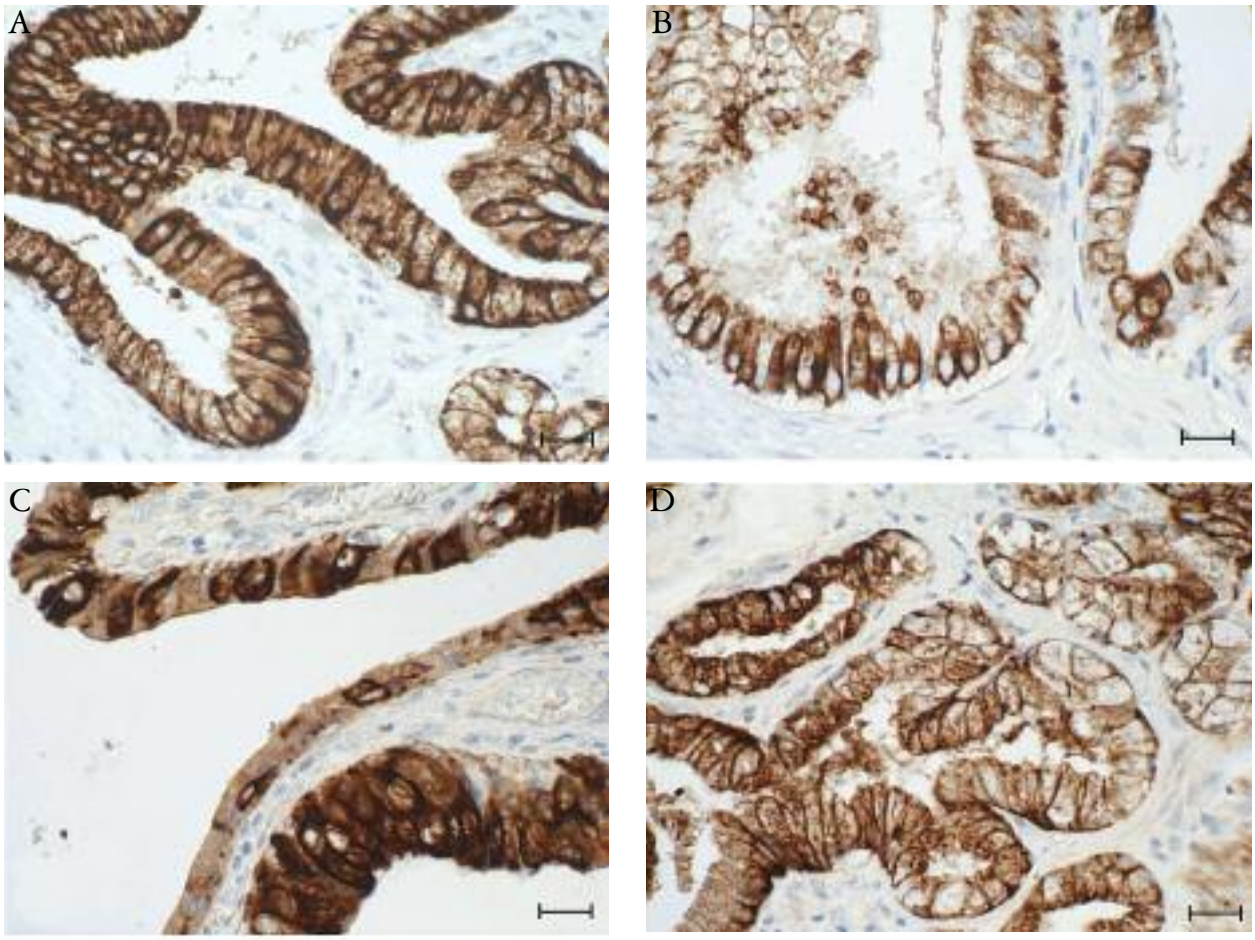

Fig. 3. CK18 expression in gallbladder mucosa of patients with CH: (A) strong positive reaction in numerous gallbladder epithelial cells; (B) perinuclear and basolateral localisation of CK18 in columnar epithelial cells; (C) evident CK18 detection in some regenerating gallbladder epithelial cells; (D) positive reaction of CK18 in tubuloacinar glands. ABC technique. Haematoxylin counterstained. Bar = 20 μm (A-D)

Our study has confirmed expression of both CKs in most cells of gallbladder epithelia and in all patients, independently of their group. Cellular localisation has confirmed cytoplasmic expression of the two proteins, particularly intense in the perinuclear zone and on lateral cell membranes. Expression of both CKs has pertained in particular columnar cells of gallbladder while CK18 probably has been manifested also on a few brush cells. Nevertheless, we cannot confirm that CK18 has represented a specific marker of brush cells [13]. It should be added that very strong immunocytochemical expression of both CKs has involved both histologically intact and regenerating regions of gallbladder epithelium. The present studies using the modern technique of spatial visualization have permitted a precise quantitative evaluation of both CKs. The significantly higher expression of CK8 and 18 has been disclosed in gallbladder of older patients, as compared to the younger ones. It seems important that CK8 expression in older patients was higher both in patients with acute and chronic clinical symptoms as compared to respective subgroups of the younger patients. Also, higher expression of CK18 has been disclosed in older patients, as compared to the younger ones, but only those with chronic clinical symptoms. Moreover, only in the old-

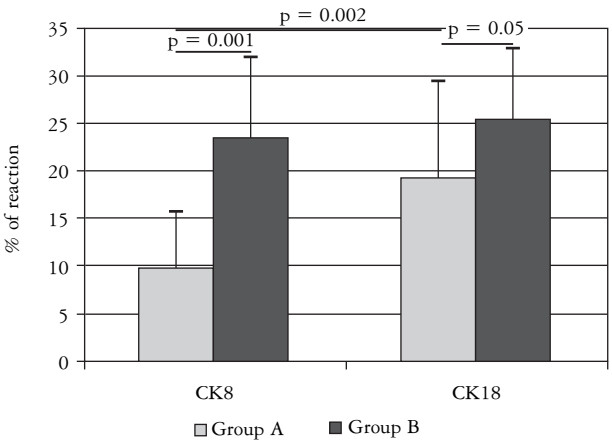

Fig. 4. Comparative expression of CK8 and 18 (% of the immunocytochemical reaction) in gallbladder mucosa in the young (A) and older patients (B) with symptomatic cholelithiasis (±SD)

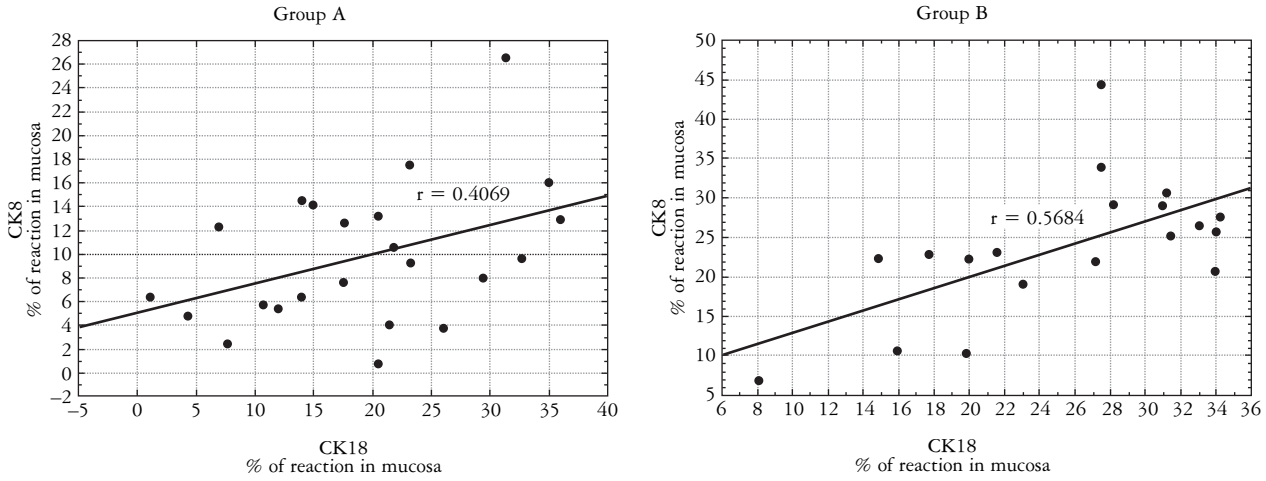

Fig. 5. Spearman's correlation between reciprocal expression of CK8 and CK18 in gallbladder mucosa of patients in group A and group B; r – Spearman's rank correlation coefficients, $p \leq 0.05$

Table II. Spearman's rank correlation coefficients (r) between CK8 and 18 expression (% of the immunocytochemical reaction) in gallbladder mucosa and selected clinical data in young (A) and older patients (B) with symptomatic cholelithiasis

Clinical data	A		B	
	CK8	CK18	CK8	CK18
BMI (kg/m^2)	0.087	0.172	–0.167	–0.405
Number of gallstones	–0.145	–0.218	0.204	–0.084
Width of gallbladder wall (mm)	0.096	0.211	–0.067	0.156
Grading (G1)	–0.022	–0.032	–0.020	–0.250
Grading (G2)	–0.172	–0.072	–0.302	–0.241
WBC ($\times 10^9$/l)	–0.206	0.272	–0.427	–0.312
PLT ($\times 10^9$/l)	0.121	0.127	–0.021	0.239
Bilirubin (mg/dl)	–0.162	0.072	–0.257	–0.496*

*BMI – body mass index; WBC – white blood cells; PLT – platelets; *indicates values of r significant at $p < 0.05$*

Table III. Comparative expression of CK8 and 18 (% of the immunocytochemical reaction) as related to clinical course at the time of admission to the hospital (±SD)

Cytokeratins	Clinical course	A	B
CK8	Acute	10.05 ±8.29	24.18 ±10.37
		p = 0.002	
	Chronic	9.75 ±4.62	22.46 ±6.92*
		p = 0.001	
CK18	Acute	18.97 ±12.29	23.43 ±7.98
		p = 0.536	
	Chronic	19.31 ±9.81	26.98 ±7.26
		p = 0.048	

**p = 0.029 in CK8 expression between acute and chronic course of the disease in group B*

er patients with acute clinical symptoms, as compared to patients with chronic symptoms, has a significant prevalence of CK8 expression been noted. No such difference could be noted in the group of young patients.

Our results suggest an intensified expression of both CKs and their role in cases of a more intense epithelial regeneration, particularly in the group of older patients as compared to the more "physiological" ex-

pression of the CKs in younger age. In parallel, the group of younger patients has manifested an almost twofold higher expression of CK18 as compared to expression of CK8, which suggests a different role of both CKs in lithogenesis in younger patients. Interestingly, despite the quantitative differences in expression of the two CKs in young and older patients with CH, a strong direct relationship has been demonstrated between reciprocal expression of the two CKs, and this has been true in both groups.

Analysis of histopathological and other clinical data in the studied groups of patients has shown that expression of neither CK correlated with inflammatory activity in the gallbladder or other results of laboratory tests significant for surgical practice. The only demonstrated correlation, a negative correlation, has been established for expression of CK18 and total bilirubin serum level of group B patients. Other authors [23, 24] have found that CKs play a role mainly in hepatic diseases but no such studies have been performed in inflammatory diseases of gallbladder. In the mouse model of cholestasis, augmented expression of CK8 and 18 was detected in hepatocytes. Results of the studies point to a potential role of biliary acids in stimulation of CK production and their phosphorylation in the mouse liver [27]. As far as the relationship between CK18 and bilirubin concentration is concerned, studies on porcine liver have shown that just this CK manifests affinity to bilirubin and may play a role as a membranous reservoir of the protein in cases of transport and/or secretion of bile pigments in the liver [28]. In order to verify applicability of this way of thinking to human liver, functional studies should be performed in gallbladder using an *in vitro* model.

In summary, a positive correlation between reciprocal expression of the two CKs may confirm a cytoprotective role of the two proteins in both groups of patients with symptomatic CH. Significantly higher expression of CK18 than CK8 in younger patients suggests a different role of CK8 and 18 in lithogenesis in this group.

Supported by a grant from the Rector of Poznan University of Medical Sciences, Poznań, Poland (no. 501-02-02229373-05360-50502).

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