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Expression of the DNA Mismatch Repair Proteins (hMLH1 and hMSH2) in Infiltrating Pancreatic Cancer and Its Relation to Some Phenotypic Features

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DNA mismatch repair system defects cause microsatellite instability (MSI) and form an alternative pathway in cancer development. Germline mutations of DNA mismatch repair genes account for hereditary nonpolyposis colorectal cancer, which has a different morphology and biology than sporadic cancers. MSI has also been found in sporadic neoplasms and some inflammatory conditions (chronic pancreatitis, ulcerative colitis). The purpose of the present study was to evaluate the expression of hMLH1 and hMLH2 proteins in infiltrating pancreatic cancer and to find out whether there is a relationship between some phenotypic manifestations and expression of MMR genes. We studied 30 cases of infiltrating pancreatic cancer and apart from hMLH1 and hMLH2 expression cytokeratin 7 and chromogranin were measured as markers of ductal and endocrine differentiation, respectively. All ductal pancreatic cancers expressed cytokeratin 7. In most cases the expression was strong, present in 50 - 100% of cells in moderately differentiated cancers and in 80 - 100% of cells in poorly differentiated cancers. Chromogranin expression was seen in 5 moderately differentiated cancers and in 6 poorly differentiated cancers (up to 20% of positive cells). In all cases DNA mismatch repair genes expression was present. Conclusion: Ductal pancreatic carcinomas express hMLH1 and hMLH2 proteins irrespective of their differentiation. The expression of cytokeratin 7 is typical of ductal pancreatic carcinoma and its level is related to cancer differentiation. Some ductal pancreatic carcinomas irrespective of their differentiation show the expression of chromogranin, which is associated with the expression of hMSH2 gene.

Introduction

DNA replication errors, "side effects" of DNA polymerase activity (DNA mismatches) can be corrected by DNA mismatch repair system. The characteristic feature of the cell genome with faulty DNA mismatch repair is microsatellite instability (MSI), and the phenotype is denoted as RER+ (mutator phenotype) [6]. This mutator phenotype accounts for the multiple mutations resulting in multistage carcinogenesis [17]. The DNA mismatch repair system consists of at least six genes: hMLH1, hMSH2, hMSH3, hMSH6, hPMS1, hPMS2. Inactivation of hMLH1 and hMSH2 genes occurs most frequently in the process of carcinogenesis [16].

Germline MMR mutation accounts for 80 - 90% of Lynch syndrome cases (hereditary nonpolyposis colorectal cancer). These tumours, making up 5 - 10% of colorectal cancers, in contrast to sporadic carcinomas have a better prognosis, occur in younger subjects, involve predominantly the right side of the colon, are diploid, rarely show loss of heterozygosity (LOH) and have a characteristic morphological pattern. Furthermore subjects with HNPCC are at risk of a variety of other cancers [14, 19, 20].

MSI has also been found in sporadic colorectal, endometrial, pancreatic, gastric and ovarian cancers, although the level of MSI is rather low in most sporadic neoplasms [2, 5, 8, 9, 10, 13, 16].

Microsatellite instability in pancreatic cancers varies from 0 to 67% [8, 11, 23]. Brentnall also found the presence of MSI in two or more loci in patients with chronic pancreatitis [3].

Thibodeau and Marcus demonstrated that immunohistochemistry can be used to identify MSI from the expression of hMLH1 and hMSH2 genes. The sensitivity and specificity of the test was 97% and 100%, respectively [22, 25]. The use of immunohistochemistry offers a relatively rapid method for prescreening tumours for defects in the expression of MMR genes.

As colorectal cancers show a relationship between MMR defect and cancer phenotype, a question arises whether a similar association exists in pancreatic cancer, that is whether the morphological pattern of cancer can provide information on DNA repair status.

The purpose of the present study was to investigate the expression of MMR genes (hMLH1, hMSH2) in 30 cases of infiltrating pancreatic cancer. We also sought to answer whether there is a relationship between the expression of MMR genes and some phenotypic manifestations of pancreatic cancer.

TABLE 1

Immunohistochemical markers in moderately differentiated carcinomas

Case No	CK 7	Chromogranin	hMSH2	hMLH1
1.	100	0	40	10
2.	100	0	20	15
3.	100	20	20	15
4.	100	0	15	20
5.	90	0	20	50
6.	95	0	15	15
7.	100	10	30	15
8.	50	1	40	25
9.	100	1	50	20
10.	100	10	30	20
11.	100	0	15	15
12.	100	0	20	15

TABLE 2

Immunohistochemical markers in poorly differentiated carcinomas

Case No	CK 7	Chromogranin	hMSH2	hMLH1
1.	80	10	30	10
2.	100	0	20	15
3.	80	0	40	30
4.	80	10	40	5
5.	80	0	30	15
6.	80	10	30	0
7.	100	0	1	5
8.	95	1	50	20
9.	100	1	30	20
10.	100	0	30	35
11.	100	0	15	35
12.	90	10	30	20
13.	100	0	30	20

TABLE 3

Histological types and immunohistochemical markers in other than ductal carcinomas

Case No	Histology	CK 7	Chromogranin	hMSH2	hMLH1
1	Nondifferentiated carcinoma	80	0	30	30
2	Adenosquamous carcinoma	90	0	50	35
3	Anaplastic large cell carcinoma	30	0	20	0
4	Carcinoma in IPMT*	80	0	20	10
5	Cystadenocarcinoma	70	0	10	0

*Intraductal Pancreatic Mucinous Tumor

Material and Methods

The study population consisted of 30 patients undergoing total or partial pancreatectomy due to infiltrating pancreatic cancer (24 men, mean age 63.5 years and 6 women, mean age 59 years). In all cases tissue for histopathological examination was obtained from areas of infiltrating carcinoma and if possible from intact pancreatic parenchyma. The tissue was fixed in 10% buffered formalin and stained with hematoxylin and eosin. Histological examination included typing of infiltrating cancer and its grading (grades 1 - 3). Immunohistochemically we examined:

- signs of endocrine differentiation (immunohistochemical staining for chromogranin A) as compared with epithelial differentiation (immunohistochemical staining for cytokeratin 7);
- expression of hMSH2 and hMLH1 mismatch repair genes.

Immunohistochemical staining was performed using reagents from Santa Cruz Biotechnology Inc. USA, according to DAKO Optimised Staining System Microwaving Preparation using TechMate Horizon manufactured by LJL Biosystem Inc. (BSA modified method):

- hMSH2 (N-20) rabbit polyclonal antibody at the dilution 1:50,
- hMLH1 (N-20) rabbit polyclonal antibody at the dilution 1:50 (before incubation with both primary antibodies N-20 sections were immersed in boiling citrate buffer (pH 6) in a microwave oven with two changes of buffer for 5 minutes each.

Sections were counterstained in Harris hematoxylin and mounted with DAKO glycergel. Epidermis and sweat gland cells served as a positive control for all reactions. In all cases a negative control was also used, it included all the stages of the procedure except primary antibody. The results of immunohistochemical reactions were expressed as percentages of positive cells. In each case at least 500 nuclei were evaluated.

Cancers were considered to demonstrate inactivation of hMSH2 and hMLH1 when there was complete absence of detectable nuclear staining of neoplastic cells. Intact nuclear

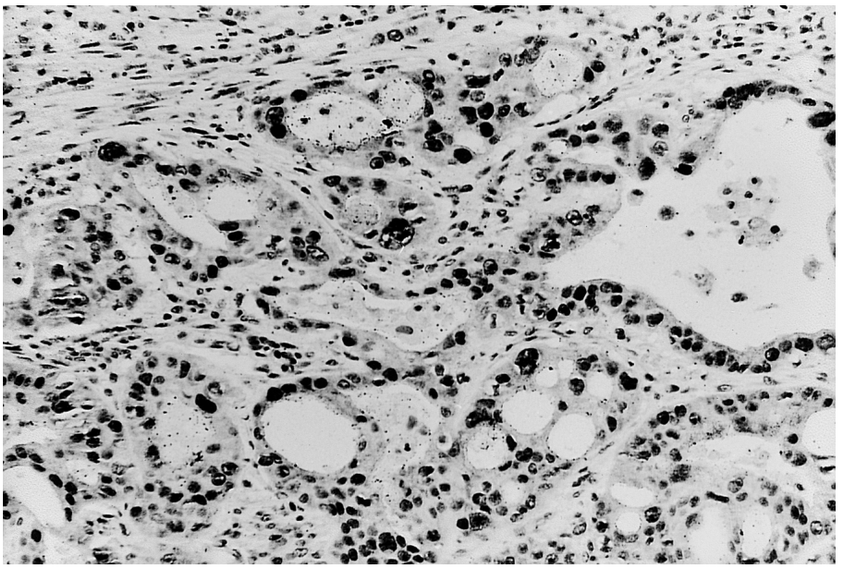

Fig. 1. Immunohistochemical expression of hMSH2 protein in poorly differentiated pancreatic carcinoma. Magn. 165x.

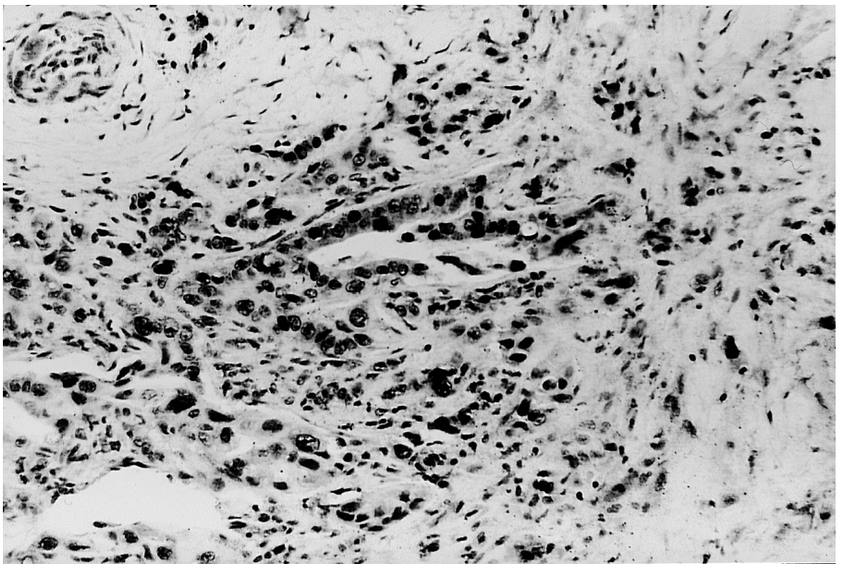

Fig. 2. Immunohistochemical expression of hMLH1 protein in poorly differentiated pancreatic carcinoma. Magn. 165x.

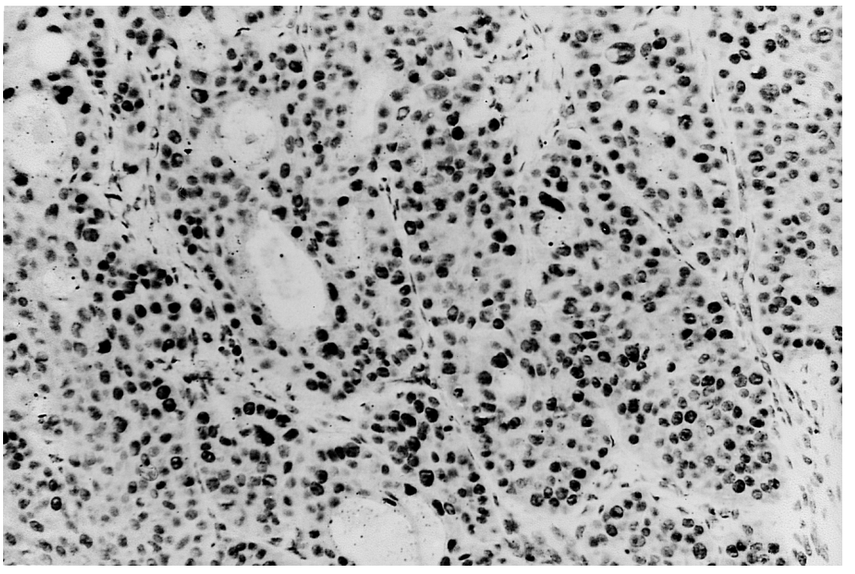

Fig. 3. Immunohistochemical expression of hMSH2 protein in adenosquamous carcinoma. Magn. 165x.

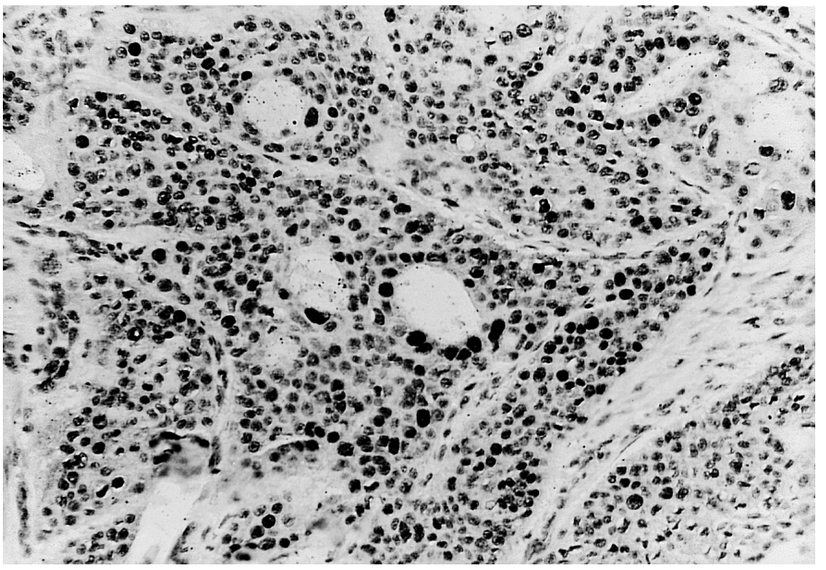

Fig. 4. Immunohistochemical expression of hMLH1 protein in adenosquamous carcinoma. Magn. 165x.

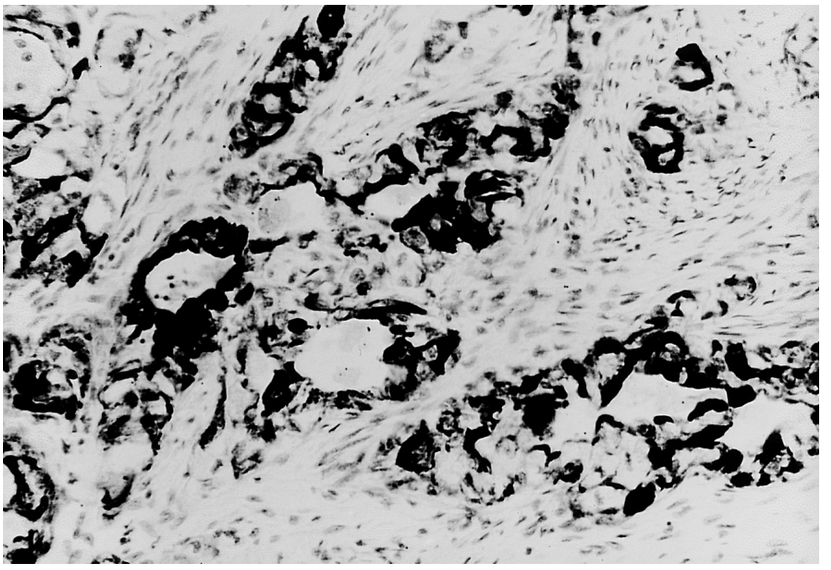

Fig. 5. Cytokeratin 7 expression in moderately differentiated pancreatic carcinoma. Magn. 165x.

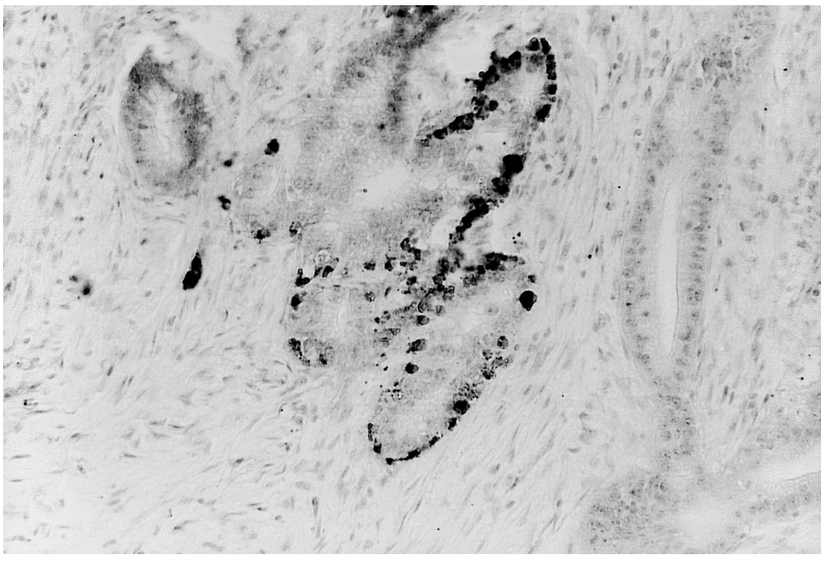

Fig. 6. Chromogranin expression in moderately differentiated pancreatic carcinoma. Magn. 165x.

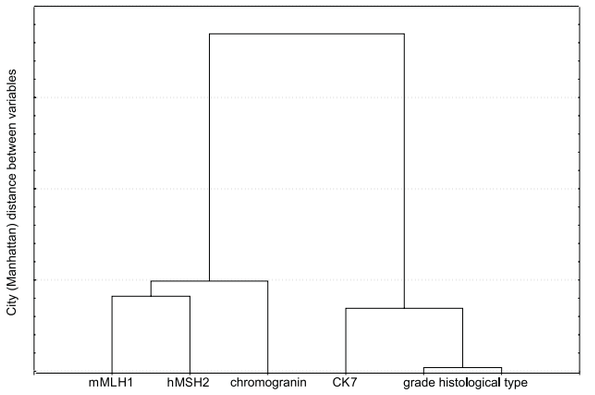

Fig. 7. The tree showing relationships between variables.

TABLE 4

Spearman rank correlation coefficient

Variables			R	p
grade	&	CK7	-0.54	0.002
grade	&	chromogranin	-0.21	0.267
grade	&	hMSH2	0.06	0.751
grade	&	hMLH1	-0.11	0.555
CK7	&	chromogranin	-0.04	0.826
CK7	&	hMSH2	-0.19	0.315
CK7	&	hMLH1	0.20	0.292
hMSH2	&	chromogranin	0.41	0.026
hMLH1	&	chromogranin	-0.12	0.517
hMSH2	&	hMLH1	0.30	0.106

staining of adjacent nonneoplastic epithelium, stromal cells or lymphocytes served as an internal control. For testing for correlations between variables, Spearman rank coefficient was used. For testing for differences between the means, Mann-Whitney U test was used. Relationships between categorised variables were tested by Pearson's χ^2 method. Relationships between variables were also studied by an agglomeration algorithm with a tree-based method (city distances, full linkage).

Significance level was set to 0.05, whereas 0.05>p>0.1 is referred as marginally significant.

Results

The study population of 30 infiltrating cancers was divided into three subgroups: moderately differentiated cancers (G2 - 12 cases), poorly differentiated cancers (G3 - 13 cases) and other than ductal cancers - 5 cases (Table 3 provides details on histological typing).

Table 1 summarises the expression of cytokeratin 7, chromogranin, hMSH2 and hMLH1 gene products (% of positive cells) in a group of 12 moderately differentiated pancreatic cancers.

Table 2 summarises the expression of cytokeratin 7, chromogranin, hMSH2 and hMLH1 gene products (% of positive cells) in a group of 13 poorly differentiated pancreatic cancers.

Table 3 summarises the expression of cytokeratin 7, chromogranin, hMSH2 and hMLH1 gene products (% of positive cells) in a group of the remaining 5 pancreatic cancers.

All cancers expressed hMLH1 and hMSH2 proteins. In all cancers from the group 1 both genes were expressed, in group 2 in one case only the expression of hMSH2 gene was seen and in group 3 - in 2 cases (Figs. 1 - 4).

All ductal pancreatic cancers expressed cytokeratin 7. In most cases the expression was strong, present in 50 - 100% of cells in moderately differentiated and in 80 - 100% of cells in poorly differentiated cancers. Strong expression of cytokeratin 7 was also observed in group 3 except large cell anaplastic carcinoma, in which cytokeratin 7 was expressed only in 30% of cells (Fig. 5).

Chromogranin expression as a marker of endocrine differentiation was seen in 5 moderately differentiated cancers and in 6 poorly differentiated cancers. The percentage of positive cells varied from 1 to 20% (Fig. 6). There was no chromogranin expression in group 3.

Then hMSH2 and hMLH1 variables were categorised as follows: strong hMSH2 expression was defined as positive response in at least 20% of cell; strong hMLH1 expression was defined as positive response in at least 15% of cell. These cut-off values were accepted based upon the maximal value of the histogram. The next step was to perform Pearson's chi^2 test.

In Figure 7 the relationship between variables is shown. It is evident that expression of hMLH1 and hMSH2 is highly similar, but also an association of these markers with chromogranin expression is also present. The correlation analysis of the variables studied is shown in Table 4.

There was a significant, reverse correlation between the degree of cancer differentiation and cytokeratin 7 expression. A significant correlation between chromogranin and hMSH2 expression was also seen. However, no difference between moderately and poorly differentiated carcinomas was present (Table 5, Fig. 8).

When comparing the categorised variables, a marginally significant relationship (χ^2=3.137, p=0.07652) of chromogranin and hMSH2 expression was present. hMSH2 and hMLH1 were definitely interrelated (χ^2=5.129, p=0.02353).

Discussion

The present study was designed to search for a relationship between expression of selected DNA mismatch repair genes and some phenotypic manifestations of pancreatic

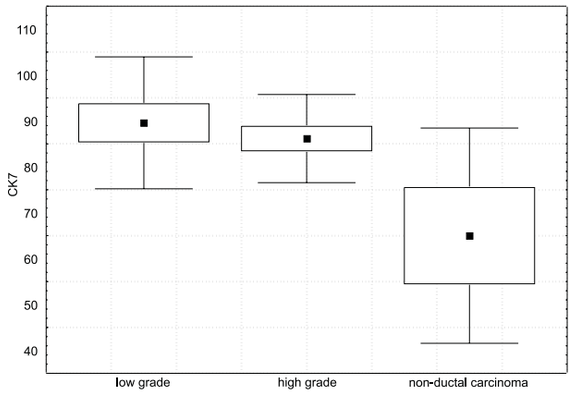

Fig. 8. CK7 expression in ductal, low and high grade, and non-ductal pancreatic carcinomas. Central point is the arithmetic mean, box is mean±standard error of mean, whisker is mean±standard deviation.

TABLE 5

Differencies of the markes studied between moderately and poorly differentiated carcinomas. U is Mann-Whitney test value; p is significance level

	U	p
CK7	54.0	0.197
chromogranin	75.5	0.892
hMSH2	62.0	0.384
hMLH1	76.5	0.935

cancer, similar to large intestinal cancer, in which genetic differences have a great impact on the morphological pattern and biology of the tumour. Cancers with DNA mismatch repair system defects have specific biological features, which can serve as predictors of the disease course and therapeutic outcome. Genetic studies of the MMR system require specialised equipment and for this reason they can be carried out in few laboratories. Therefore it is so important to find a relatively simple and cheap method for prescreening cases for further diagnosis. The use of immunohistochemistry seems to offer such a technique to study MMR gene expression [22, 25].

In colorectal cancer there are two types of neoplasms - familial and sporadic, and they have two different genetic pathways of carcinogenesis. The sporadic ones are microsatellite stable and are characterised by p53 protein accumulation. Familial cancers are characterised by microsatellite instability (MMR gene inactivation) and less common p53 accumulation. Studies show that neoplasms with DNA repair defects have a specific pathway of carcinogenesis. It was also found out that there is an inverse relationship between high microsatellite instability and p53 protein accumulation in neoplasms [1, 12, 18]. P53 gene mutations are typical of pancreatic cancer and affect its biology and behaviour. In our previous studies we demonstrated strong expression of p53 in all cases of pancreatic cancer, the stronger the lower was the histological grade of the neoplasm [26, 27]. If the above-mentioned findings are true, pancreatic cancer has to be rather microsatellite stable.

Colorectal cancers with the RER+ phenotype have a specific morphology, better prognosis and diploid karyotype [14, 19]. Goggins identified a medullary carcinoma of the pancreas characterised by poor differentiation, a syncytial growth pattern and expanding rather than infiltrating tumor borders [7]. RER- pancreatic cancers did not have these morphological features. RER+ pancreatic cancers resembled RER+ colorectal cancers except mucin production and lymphoid infiltrate, which were absent in RER+ pancreatic cancers [28]. The present study shows that ductal pancreatic cancers express hMSH2 and hMLH1 genes. This is in accordance with the findings of Ghimenti that the genetic mechanism of carcinogenesis in sporadic pancreatic cancer is not likely to be linked to microsatellite instability [8]. Only medullary pancreatic carcinoma develops in the setting of this defect. The recognition of medullary pancreatic carcinoma in routine examinations signifies MMR defect and is an indication for further genetic studies, search for other organ neoplasms and should speer investigation of the cancer incidence among the patient's relatives [28]. The risk of pancreatic cancer has been found to be increased in first-degree relatives [4, 24]. In familial pancreatic cancer the risk was increased independently of other known risk factors: alcohol consumption, smoking, pancreatitis or diabetes [4, 21]. The diagnosis of medullary carcinoma determines also treatment modality, because RER+ cancers are resistant to alkylating agents [7].

The present study and other findings indicate that all pancreatic cancers, which are not medullary, irrespective of their differentiation and histological type have intact DNA mismatch repair system. They all express cytokeratin and some of them also chromogranin. The presence of endocrine cells in ductal pancreatic cancers is a frequent phenomenon, in the present study almost half of pancreatic cancers contained cells with endocrine differentiation, which was significantly associated with expression of hMSH2 gene. A question arises whether the efficacy of the DNA mismatch repair system affects expression of markers of epithelial and endocrine differentiation. Expression of these markers in adenocarcinomas is variable - in about 44% of cases the phenotype of cancer cells is heterogeneous [15].

In summary we demonstrated that ductal carcinomas of the pancreas express the activity of mismatch repair genes (hMLH1 and hMLH2). Their expression is not related to the degree of cancer differentiation. Cytokeratin 7 as a decisive marker of ductal phenotype was significantly associated with histological grade of cancer. Chromogranin expression, relatively frequent in pancreatic ductal carcinoma was associated with the expression of hMSH2 gene.

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