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MOLECULAR DETECTION OF METASTASES IN REGIONAL LYMPH NODES IN PATIENTS WITH CANCER OF THE HEAD OF THE PANCREAS. CLINICAL RELEVANCE.

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IV LIST OF ABBREVIATIONS

Bp -	basepair
BstNI -	bacillus stearothermophilus NI (protease)
CDNA -	complementary deoxyribonucleic acid
CEA -	carcinoembrionic antigen
CK20 -	cytokeratine 20
CT -	chemotherapy
DNA -	deoxyribonucleic acid
DNTP -	deoxynucletide-3-phosphate
EDTA -	ethylendiamintetraacetat
Fig. -	Figure
JPS -	Japanese Pancreas Society
K-ras -	„Kirsten-rat sarcoma“ oncogene
LN -	lymph node (station)
MDM -	molecular detected metastases
MASA -	mutated allel-specific amplification method
mRNA -	messenger-ribonucleic acid
OS -	overall survival
PCR -	polymerase chain reaction
PFS -	progression free survival
PSA -	prostate specific antigen
Rb -	retinoblastoma gene
RFLP -	restriction fragment length polymorphism reaction
RNA -	ribonucleic acid
RO -	no residual tumor
RT-PCR -	reverse transcriptase polymerase chain reaction
Taq -	Thermus aquatiqus
TBE-Buffer -	tris-borate-EDTA buffer
TNM -	malignant tumor classification (Tumor, Nodus, Metastasis)
UICC -	Union Internationale Controle Le Cancer

1. INTRODUCTION

1.1 Pancreas cancer. Aspects of surgical treatment

Pancreatic cancer is the 14th most common cancer worldwide. Each year approximately 216 000 new cases of the disease appear. Today, no effective early diagnostic test or screening procedure is available. Five-year survival rates for pancreatic cancer patients are usually less than 5%; 80% of all patients die within one year of diagnosis [1]; 90% of pancreatic tumors are ductal adenocarcinoma; 70% of pancreatic tumors develop in the head of the pancreas [2] ; approximately 30 % of the cases are attributable to tobacco smoking while less than 8% are due hereditary syndromes [3].

The presence of metastases in regional lymph nodes, tumor size is greater than 2 cm, invasion of blood vessel walls, low grade of tumor differentiation, polyploidy and mutations in oncogenes and oncosupressors negatively influence the prognosis of the patients. This conclusion is generally accepted. Moreover, according to Ishikawa [4] the involvement of only local pancreatoduodenal lymph nodes is associated with a less dismal prognosis than the involvement of more distant lymph nodes (para-aortal, coeliac, proximal hepatoduodenal etc., see Fig. 14). There only surgical treatment with complete tumor removal (R0 - resection) may allow for some chance of long-term patient survival. Curative resection for patients with carcinoma in the head of the pancreas is an extended Whipple-Kausch procedure with regional lymphadenectomy. Still, tumor free resection margins and absence of distant metastases can be achieved in only 10-15% of all patients [5]. Despite this, postoperative morbidity remains high at 30-40% [2] and some specialized centers have achieved remarkable success in this field. Postoperative mortality has been lowered down to1,5% - 2,2% [6, 7]; 60 - 75% of patients are discharged from the hospital between the 8th and 15th day [8] ; and 5- year survival rates reach 13,8 -16,4% [9, 10]. However, more than 40% of the patients with pancreatic cancer develop local-regional recurrence within 6 months and 60-80% do so within 12 months of surgical treatment [11, 12, 13] .

The recent experience of Japanese surgeons [14, 16] shows benefits in 5-year survival (up to 28%) for patients with pancreatic head cancer who have undergone extended retroperitoneal lymphatic dissection versus those who have undergone standard dissections (up to 9 %). These results contradict reports from western investigators [17-21]. A lymphodissection begins at the lateral edge of the superior mesenteric artery and the proper hepatic artery; it involves the lymph nodes along the portal vein, the superior mesenteric vein and includes removal of the lymph nodes of the pancreatic head and of the pancreaticoduodenal region. This procedure corresponds to the R1- procedure (different from UICC R1). Removal of para-aortal and paracaval nodes and those in the region of the celiac trunk, superior mesenteric artery and the splenic artery is known as an R2-procedure. The extended lymphodissection is usually performed with parapancreatic soft tissue and neural plexus removal. The latter leads to severe, treatment resistant diarrhea attacks persisting for up to one year what approximately corresponds to median survival of these patients having metastases in distant lymph nodes. To avoid this complication the mesenterial neural plexus should be preserved at the left side of the superior mesenteric artery.

Clearly, the role of distant lymph nodes in dissemination of pancreatic cancer is of great interest. Routinely the lymph nodes are investigated after operation only histologically. A number of recent studies, where the lymph nodes were investigated using more sensitive morphological (immunostaining) and molecular (Polymerase Chain Reaction (PCR) methods, revealed that in 53-73% of the patients the isolated cancer cells were detected in histologically tumor free lymph nodes [22-24] . Some investigators also report about the clinical relevance of these findings in terms of survival prognosis [24-27]. Nevertheless, the reports are few and

the number of included patients with isolated cancer cells or molecular detected metastases (MDM) has been very limited (only 5-13 patients).

1.2 PURPOSE

In this study the regional lymph nodes of patients with adenocarcinoma of the pancreas were investigated. The markers for the MDM were K-ras mutation in codon 12 and Cytokeratin 20 expression. They were detected by means of Restriction Fragment Length Polymorphism PCR and Reverse Transcription PCR (respectively, RFLP and RT PCR).

The purpose of the study is:

1. To test the efficacy of two molecular methods: RFLP PCR for K-ras mutation in codon 12 and RT PCR for CK 20 expression, as well as their combination in molecular detection of lymph nodes metastases in patients with adenocarcinoma of the pancreas. Note: This combined method should reflect not only the appearance of tumor genetic material in the lymph node, but also its viability, due to detection of CK20 messenger RNA, which is a short-living molecule.
2. To analyze the correlation between the nodal MDM appearance and different morphological parameters of the primary tumor and lymph node status in R0 patients with adenocarcinoma of the head of the pancreas.
3. To investigate the pattern of MDM in different distant lymph nodes stations in R0 patients with adenocarcinoma of the head of the pancreas.
4. To analyze the influence of distal nodal MDM and another putative factors on progression free and overall survival rate in R0 patients with adenocarcinoma of the head of the pancreas.

2. MATERIALS AND METHODS

2.1 Patients

This study is based upon data derived from the investigation of two groups of patients. The first group included 21 patients with pancreatic cancer. These patients were treated from May 2000 to February 2001 at the Department of General Surgery of Ulm University, Ulm, Germany.

The second group included patients treated from October 2002 to August 2003 at the Center for Oncological, Endocrine and Mini-invasive Surgery, Illertissen, Germany. During that period 10 patients were diagnosed with pancreatic cancer and each underwent major surgical procedures. Three patients of this group were excluded. Of those patients excluded one was discovered to have chronic pancreatitis and canceromatose lesions of the pancreatic regional lymph nodes caused by progression of ovarial cancer. This pattern was typical for the pancreatic carcinoma lymphodissemination. One patient had pathohistologically proved neuroendocrine carcinoma of the pancreas. The remaining patient excluded had relaparatomy caused by a local recurrence of pancreatic head adenocarcinoma. This last patient has been included in the first group as number 21 (see Table 18). So only 7 patients with histlolgically proved pancreatic carcinoma from the second group were included in this study. Tissue samples were collected from 28 patients (13 males and 15 females). In the samples the primary tumors and local and distant lymph nodes were investigated. The pathohistological examination of the tissue samples was performed by different pathologists in two groups and the molecular-biological methods for detection of K-ras mutation and CK20 expression were the same.

The age of the patients varied between ages 29 and 75. The median age was 64 years. All diagnoses were histologically proven to be adenocarcinoma of the pancreas. Procedures performed: 26 pylorus-preserving Whipple's procedures, one advanced distal pancreatectomy with splenectomy and atypical liver resection and one total pancreatectomy with resection of ? of the stomach, left adrenalectomy and splenectomy. Except for one patient with carcinoma of the tail of the pancreas (Table 18, N26) and diffuse liver metastases, there was no evidence of distant metastases in any of the patients. The patient with carcinoma of the tail of the pancreas was excluded when clinical relevance was evaluated. The remaining 27 patients were R0 according to UICC Residual Tumor Classification (Table 21). There was one stage I patient, six stage II; 18 stage III and three stage IV patients (Table 18). Tumor staging was ascertained postoperatively after histological and clinical assessment according to Union Internationale Contre la Cancer (UICC) TNM Classification of Malignant Tumors, Fifth edition, 1997 (App.1).

2.1.1 Tissue samples

The tissues from the primary tumors were taken intraoperatively after their removal and frozen immediately in liquid nitrogen (t = -80ºC). The regional lymph nodes (Fig. 14) were also removed and hemisected intraoperatively, after which one half of the lymph nodes were submitted to routine histopathological examination and the other half then frozen in liquid nitrogen for further molecular biological investigation. The localization of the lymph nodes was marked according to classifications of the Japanese Pancreas Society.

2.2 Methods

2.2.1 Tissue homogenization

In order to prepare the deep-frozen tissue for nucleic acid extraction, 25 mg of the tissue was taken and ground by means of a pestle and a mortar or by using a MagNA Lyser by Roche. Paraffin-embedded tissues were vortexed and centrifuged for 5 minuets at room temperature in the presence of xylol twice. The same procedure was then repeated twice in the presence of ethanol.

2.2.2 DNA Extraction

The DNA extraction from fresh-frozen and paraffin-embedded tissues was performed with the help of a QIAamp DNA Mini Kit by "Qiagen" using the applied standard protocol for each case respectively. For centrifugation steps, Centrifuge 5415 R by Eppendorf was applied.

2.2.2.1 DNA Extraction from paraffin embedded tissues.

In order to investigate the whole lymph node, not just half of it, as well as to check the difference in results of routine histological HE staining and MB method dealing with exactly identical massive of tissue, the second half of the dissected lymph nodes were used. This half was first submitted to formalin fixing, then paraffin embedding and finally histological examination of the sections. After obtaining the results of the histological examination, the lymph nodes were cut out of the paraffin blocks and submitted to DNA extraction as described above. This method makes investigation of the whole lymph node possible (not just the half of it that had been deeply frozen (-80°C).

2.2.3 RNA Isolation

The RNA isolation from fresh-frozen tissues was carried out by means of a High Pure RNA Tissue Kit by "Roche" following the applied standard protocol. For centrifugation steps, Centrifuge 5415 R by Eppendorf was applied.

2.2.4 Measurement of Nucleic Acid Concentration

The yield concentrations of the nucleic acids were measured by photometric method (wave length 260nm) by means of Biophotometer by Eppendorf. Nucleic acid solution (60µl) was used for the testing. In each case the so called ratio (260/280), reflecting the purity of the nucleic acid solution was between 1,7 and 2,0.

2.2.5 Polymerase Chain Reaction. Control with GAPDH.

The polymerase chain reaction (PCR) technique, invented in 1985 by Kary B. Mullis makes it possible to multiply a scarce sample of DNA up to millions of copies. The technique is based upon numerous repeats of certain DNA fragment replication. The repeats are named cycles. The DNA fragment is limited by so called primers (20-30 base pairs long oligonucleotides), which bind to DNA after it is split. The DNA split is the 1st step of the cycle; the primers' binding is the second step; and the DNA-polymerase activation (the replication itself) is the third step. The DNA replication is possible only in the presence of a sufficient quantity of deoxynucliotide-3-phosphate (dNTP's). These serve as a building

material. Then the newborn double-stranded DNA enters the next cycle. Each of the 3 steps demands a certain temperature for a certain period of time.

In this study the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene detection was used as an intrinsic control of the effectiveness of PCR. GAPDH is a glycolytic enzyme (EC 1.2.1.12) that catalyses the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate. It has been shown to interact with various elements of the cytoskeleton and to be expressed ubiquitously.

2.2.6 Visualisation of the PCR products. Gel electrophoresis.

The PCR-product was separated by electrophoresis in 2% agarose gel.

In order to prepare the agarose gel, 2 g of agarose was added to 100 ml of 0,5 Tris-Borat EDTA (TBE) Buffer solution and boiled in a microwave oven. Then the solution was cooled to 60° C and 4 µl of Ethidium bromide was added. Afterwards the horizontal gel-chamber was filled with the solution for further polymerization. PCR product in the amount of 18 µl was mixed with 2 µl of 2×Loading buffer and pipetted into the cells of polymerized gel. One of the cells was filled with 100bp marker. The electrophoresis was carried out with the voltage set at 120 for 90 minutes. Afterwards the gel was exposed to UV light (?=312 nm) with the result that the ethidium bromide bound to PCR products fluoresced. This fluorescence was documented on film by means of a Polaroid camera.

2.2.7 Enrichment PCR for detection of K-ras mutation in codon 12.

K-ras protein (molecular weight 21.000) is an enzyme (GTP-ase), which plays a central role in signal transduction from tyrosinekinase. It is involved in activation of transcription factors that are induced by external stimulation (by growth factors and cell differentiation factors). In its normal state this activity is periodical; however, in case of mutation the protein becomes permanently active. This permanently active state leads to deformation of cell membranes, loss of contact inhibition and substrate-specific growth. The frequency of K-ras gene mutation in pancreatic cancer cells is 80% - 95%.

In order to detect the point mutation in codon 12 in K-ras gene, the Restriction Fragment Length Polymorphism (RFLP) – PCR method is used. The method consists of repeated PCR step followed by a step of restrictase digestion (RFLP).

I step PCR:	primer 1	5’-CAT GAA TAT AAA CTT GTG GTA GTT GGA **C**CT - 3’
	primer 2	5’-TCA AAG AAT GGT CCT GGA CC- 3’
II step PCR:	primer 1	5’-CAT GAA TAT AAA CTT GTG GTA GTT GGA CCT - 3’
	primer 3	5’-TAA TAT GTC GAC TAA AAC AAG ATT TAC CTC-3’

The first PCR step is followed by the first digestion with restriction enzyme BstN1 aimed at palindrome formed only in non-mutated (wild type) DNA sequence in codon 11 and 12. The palindrome sequence is formed after the first PCR by a change of guanine upon cytosine in primer 1, in position 11. In all the mutated genes the palindrome sequence is not formed. The digestion is performed at 60 º C for three hours. After the first digestion the non-mutated DNA is deprived of the lot complementary for primer 1. Therefore in the second PCR only the DNA of mutated genes is amplified. The amplified mutated DNA segment (final PCR product) is 135 base pairs long. The second digestion step is performed to remove aberrant sequences, which may not have been restricted during the first digestion, and which could create visual interference. The protocol of the reaction is represented in Table 1.

Table 1. Protocol of RFLP-PCR for codon 12 K-ras mutation detection.

PCR Step I

Volume / µl	Components
2	DANN, in concentration of 25ng/µl
2	PCR-Buffer 10*
0,5	Desoxynucleotide Triphosphate (dNTP's), in concentration 10mM/l
0,5	Primer 1, in concentration of 5 pmol/ µl
0,5	Primer 2, in concentration of 5 pmol/ µl
1	Taq- polymerase, activity 1 Unit/ µl
13,5	Aqua bidestillata

Digestion I

Volume / µl	Components
2	PCR product
2	Reaction buffer 10*
1	Restrictase BstnI, activity 5 Units/ µl
15	Aqua bidestillata

PCR Step II

Volume / µl	Components
2	Restriction product
2	PCR-Buffer 10*
0,5	Desoxynucleotide Triphosphate (dNTP's), in concentration 10mM/l
1	Primer 3, in concentration of 5 pmol/ µl
1	Primer 4, in concentration of 5 pmol/ µl
1	Taq- polymerase, activity 1 Unit/ µl
12,5	Aqua bidestillata

Digestion II

Volume / µl	Components
10	PCR product
2	Reaction buffer 10*
2	Restrictase BstnI, activity 5 Units/ µl
6	Aqua bidestillata

The PCR and digestion was carried out in "Mastercycler gradient" by Eppendorf.
The PCR step I consists of 15 cycles and the PCR step II– of 30 cycles. The cycles are equal:

Cycler programm

Cycle N1 in PCR I and II	denaturation	96º C	2 min
	annealing	55º C	1 min
	DNA- Synthesis	72º C	1 min
Cycles N 2-15 (for PCR I) and N2-30 (for PCR II)	denaturation	96º C	1 min
	annealing	55º C	1 min
	DNA- Synthesis	72º C	1 min
Final DNA- Synthesis (for PCR I and PCR II)		72º C	10 min

As negative control for K-ras mutation determination the DNA extracted from HT-29 cell line was used. For positive control, DNA from SW-480 cell line was used.

2.2.8. Reverse Transcriptase (RT) PCR for detection of CK20 expression.

Cytokeratine 20 is structural protein of normal epithelial cells. This protein is not affected by malignant transformation of a cell; it is also evidenced in cancer cells. That makes it possible to identify the MDM in the lymphatic nodes, since normal lymph nodes tissues, being of mesenchimal origin, do not express CK 20. The frequency of CK 20 expression in different epithelial tumors varies from 40% to 90%.

The search for CK 20 expression was carried out on messenger-RNA (mRNA) level. In order to preserve the genetic information the unstable RNA was transcribed into complementary DNA (cDNA) by RT PCR. This has been done according to the protocol of the SuperScript II Kit by Invitrogen. The cDNA was further used for nested PCR – two step PCR with specific primers for each step:

I step: primer 1 'outersence' 5'-CGT CTA ACA GTC GAA GCT GAT CTC- 3'
primer 2 'outerantisence' 5'-TCG GGC GTT CCA TGT TAC TC - 3'
II step : primer 3 'innersence' 5'- AAG CAT CTG GGC AAC ACT GTC A – 3'
primer 4 'outersence' 5'-AAC GGG CCT TGG TCT CCT CTA-3'

The protocol for the nested PCR is represented in Table 2.

Table 2. Protocol of nested PCR reaction .

I Step PCR

Volume / µl	Components
10	cDNA, in concentration of 250ng/µl
2,5	PCR-Buffer 10*
0,5	Desoxynucleotide Triphosphate (dNTP's), in concentration 10mM/l
1	Primer 1, in concentration of 5 pmol/ µl
1	Primer 2, in concentration of 5 pmol/ µl
0,5	Taq- polymerase, activity 1 Unit/ µl
9,5	Aqua bidestillata

II Step PCR

Volume / µl	Components
10	I Step PCR product
2,5	PCR-Buffer 10*
0,5	Desoxynucleotide Triphosphate (dNTP's), in concentration 10mM/l
1	Primer 3, in concentration of 5 pmol/ µl
1	Primer 4, in concentration of 5 pmol/ µl
0,5	Taq- polymerase, activity 1 Unit/ µl
9,5	Aqua bidestillata

Cycler programm

Cycle N1 in PCR I and II	Denaturation	95º C	6 min
	Annealing	60º C	30 sec
	DNA- Synthesis	72º C	1 min
Cycles N 2-15 (for PCR I) and N2-30 (for PCR II)	Denaturation	95º C	1 min
	Annealing	60º C	30 sec
	DNA- Synthesis	72º C	1 min
Final DNA- Synthesis (for PCR I and PCR II)		72º C	10 min

The final nested PCR product was 313 base pairs long oligonucleotide. RNA-isolated from Yorkat cell line was used as negative control and from HT-29 cell line – as positive control.

2.2.9 Statistical analysis

As a basis for the analysis of the data, the "MedCalc", version 5.00.012 ©1993/98 was employed. The overall survival rates (as for October14th , 2003) were calculated for patients with carcinoma of the head of the pancreas using Kaplan-Meier method with no correction made for age. Differences with respect to survival were investigated by means of a log-rank test. The explorative p-value was considerable, when lower than 0.05. One patient was lost from follow up in the 20th month after the operation and was censored. The follow up started right after the operation and patients with postoperative mortality were included. The factors influencing MDM were tested for independence by a $?^2$ – test.

3. RESULTS

3.1 K-ras mutation and CK20 expression in primary tumors

In the 28 patients included in this study, K-ras positive primary tumors were found in 22 (79%) and CK20 in 14 (50%) of the patients. All the primary tumors expressing CK20 were also K-ras positive.

3.2 Histological and molecular findings in the lymph nodes. Sensitivity and specificity of molecular-biological methods. Relation to histological method.

Altogether there were 531 lymph nodes histologically investigated in this study. Of these 226 (42%) were regional lymph nodes. These were submitted only to histological investigation. The rest of the 305 lymph nodes were both hemisected and tested histologically. They were also tested for the presence of K-ras mutation and 239 of these were tested for CK20 expression.

In the 28 patients of both groups, the histological examinations showed tumor invasion in 31 of 241 (12.9%) regional and in 32 of 290 distant (11.0%) lymph nodes.

Application of molecular-biological (molecular, MB) methods (K-ras and CK20 together) increased these indices up to 25.9% for the distant lymph nodes in all patients.

In the 28 patients the general number of the lymph nodes containing tumor cells detected by any of applied methods was 106 of 531 (20%) (Table 3). In the first group there were15 histologically positive distant lymph nodes and all of them appeared to be positive for at least one molecular marker. In the second group the number of all molecular positive lymph nodes was 20.This number did not cover the quantity of histologically positive - 25 lymph nodes. So for the more complete evaluation of lymph nodes involvement in cancer dissemination we further used the index “any of the methods”. In this, we considered nodal involvement, if the presence of tumor cells were proved by at least one of the methods used (see Table 3, 9).

Table 3. Comparison of positive results of histological and molecular investigation of the lymph nodes of 28 patients with adenocarcinoma of pancreas head .

Lymph nodes	Histology	K-ras	CK 20	Any of the methods	Total number
Regional	12.9% *(31/241)*	40.0% *(6/15)**	n.i	12.9% *(31/241)*	45% *(241)*
Distant	11.0% (32/290)	15.9% *(46/290)*	15.6% *(37/237)*	25.9% *(75/290)*	55% *(290)*
Regional + distant	11.9% *(63/531)*	17.1% *(52/306)*	15.6% *(37/237)*	20.0% *(106/531)*	100% *(531)*

* - Investigated only the 2nd group

To evaluate the concordance of the molecular methods with the histological method in patients with K-ras positive primary tumors we used data from the 22 patients with K-ras positive primary tumors (Table 4.a). The concordance (number of true positive results) was calculated as a percentage proportion of the number of lymph nodes positive for at least one

molecular marker among histologically positive lymph nodes (LN). The concordance reached 96.65%. The distribution of molecular findings in lymph nodes is represented by Figure 2.

Table 4.a. Distribution of molecular findings among histologically positive and negative lymph nodes in patients with K-ras positive primary tumor.

Lymph nodes	K-ras positive	K-ras negative	CK 20 positive	CK 20 negative	K-ras or CK 20 pos	K-ras or CK20 "-"
Histologically positive	**concordance 89.7%** *(24/29)*	False negative 10.3% *(3/29)*	**concordance 38.5%** *(5/13)*	False negative 61.5% *(8/13)*	**concordance 96.65 %** *(28/29)*	False negative 3.35% *(1/29)*
Histologically negative	11.4% *(25/220)*	88.6% *(195/220)*	17,5 % *(21/120)*	82.5% *(99/120)*	16.8 % *(37/220)*	83.2% *(183/220)*
Total	20.5% *(51/249)*	79.5% *(198/249)*	19.5% *(26/133)*	80.5% *(107/133)*	26.1% *(65/249)*	73.9% *(184/249)*

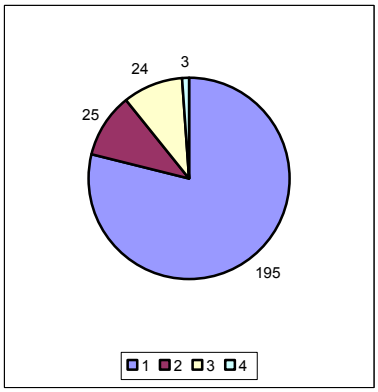

Fig. 1.a Distribution of K-ras mutation among histologically "+" and "-" LN (n=249).

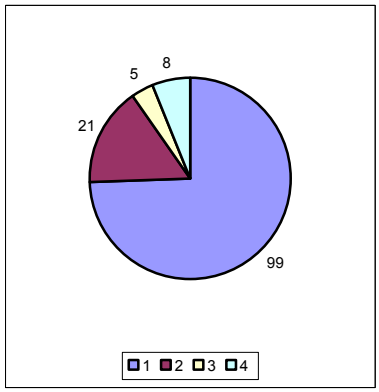

Fig. 1.b Distribution of CK20 expression among histologically "+" and "-" LN (n=133).

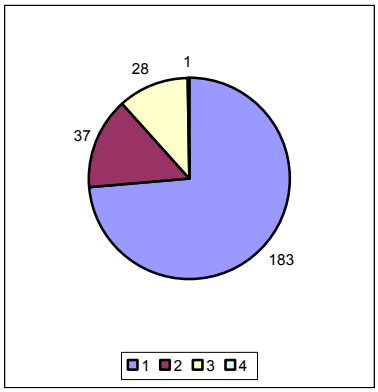

Fig. 1.c Distribution of MB "+" and "-" among histologically "+" and "-" LN (n=249).

1. K-ras "-" among histol "-" LN
2. K-ras "+" among histol "-" LN
3. K-ras "+" among histol "+" LN
4. K-ras "-" among histol "+" LN

1. CK20 "-" among histol "-" LN
2. CK20 "+" among histol "-" LN
3. CK20 "+" among histol "+" LN
4. CK20 "-" among histol "+" LN

1. MB "-" among histol "-"LN
2. MB "+" among histol "-" LN
3. MB "+" among histol "+" LN
4. MB "-" among histol "+" LN

Fig. 1. Distribution of molecular findings among histologically positive and negative lymph nodes in patients with MB positive primary tumor of the both groups.

In the patients with K-ras positive primary tumors there were 133 lymph node samples that were investigated by all methods (histology, K-ras and CK20 markers). In 19 of the cases (14%) the concordance of positive CK20 and K-ras markers was concurrent (Fig. 2). In four of the cases both positive molecular markers were concordant with positive pathohistological results. The general number of histologically positive lymph nodes among investigated by both molecular methods was 13 (Table 4).

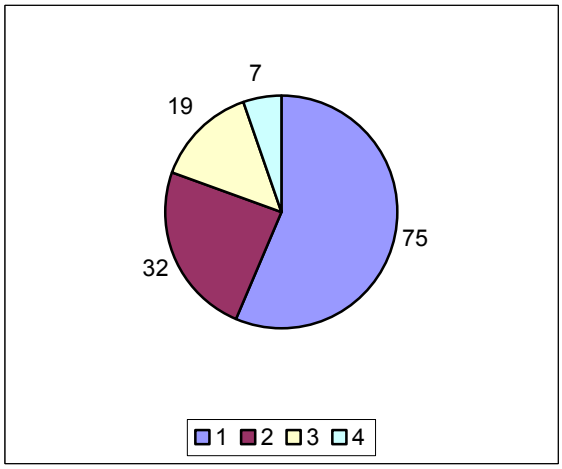

Fig. 2. K-ras and CK 20 markers in patients with K-ras positive primary tumor, n=133.

1- number of samples negative by both markers (n=75)
2- K-ras positive samples (n=32)
3- samples with concordant positive K-ras and CK20 (n=19)
4- CK20 positive samples (n=7)

In Table 4.b. the concordance of molecular and histological results is calculated for all the patients (including the patients with K-ras negative primary tumors) and totalled 70,0%.

Table 4.b. Distribution of molecular findings among histologically positive and negative lymph nodes in all patients.

Lymph nodes	K-ras positive	K-ras negative	CK 20 positive	CK 20 negative	K-ras or CK 20 pos.	K-ras or CK20 "-"
Histologically positive	**Concordance 65.0%** *(26/40)*	False negative 35.0% *(14/40)*	**concordance 36.4%** *(8/22)*	False negative 63.6% *(14/22)*	**concordance 70.0%** *(28/40)*	False negative 30.0% *(12/40)*
Histologically negative	9.8% *(26/265)*	90.2 % *(239/265)*	13.4% *(29/216)*	86.6% *(187/216)*	16.6% *(44/265)*	83.4% *(221/265)*
Total	17.0% *(52/305)*	83.0% (253/305)	15.6% *(37/237)*	84.4% *(201/237)*	23.6% *(72/305)*	76.4% *(233/305)*

The distribution of molecular findings among lymph nodes is represented by Fig. 3.

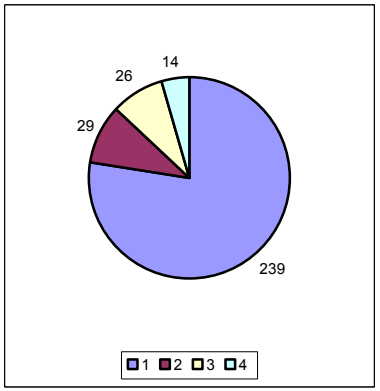

Fig. 3.a Distribution of K-ras mutation among histologically "+" and "-" LN (n=305)

1. K-ras "-" among histol "-" LN
2. K-ras "+" among histol "-" LN
3. K-ras "+" among histol "+" LN
4. K-ras "-" among histol "+" LN

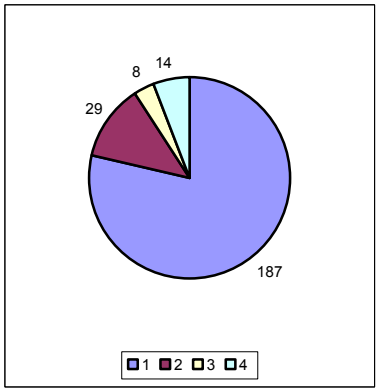

Fig. 3.b Distribution of CK20 expression among histologically "+" and "-" LN (n=237).

1. CK20 "-" among histol "-" LN
2. CK20 "+" among histol "-" LN
3. CK20 "+" among histol "+" LN
4. CK20 "-" among histol "+" LN

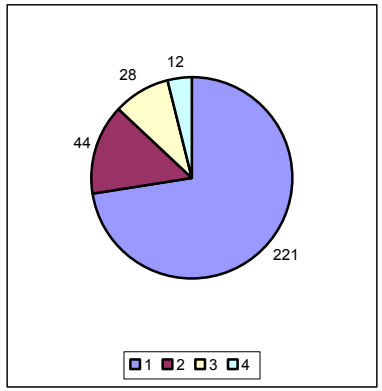

Fig. 3.c Distribution of MB "+" and "-" among histologically "+" and "-" LN (n=305).

1. MB "-" among histol "-" LN
2. MB "+" among histol "-" LN
3. MB "+" among histol "+" LN
4. MB "-" among histol "+" LN

Fig. 3. Distribution of MB findings among histologically positive and negative lymph nodes in all patients.

3.3 MDM in local and distant lymph nodes in patients with pancreatic cancer.

Patient 26 (Table 18) with pancreatic tail carcinoma was excluded when the pattern of lymph nodes involvement for patients with pancreatic head cancer evaluated.

In the remaining 27 patients with adenocarcinoma of the head of the pancreas involved in the study, the involvement of lymph nodes in cancer dissemination was proven in 25 of these patients (93%). This was proven by a combination of histological and molecular methods. The term "involvement" here considers both lymph node metastases and molecular detected metastases (MDM). The MDM was diagnosed when any of molecular markers (K-ras or CK20) was positive.

Histological examinations showed 8 patients from the 27 as pN_0. Molecular investigation also showed 8 patients who had no evidence of lymph nodes' MDM . However, those methods taken together showed only 3 patients with no signs of nodal involvement.

Local lymph node metastases were present in 18 patients (Table 5). Distant (N2, according to JPS) lymph nodes were involved in 9 patients according to histological examination, in 20 patients according to molecular investigation and in 21 patients when evaluation was made combining all methods. Among the 21patients determined by combining methods, there were 6 patients with only distant lymph nodes involvement having local histologically proved tumor free lymph nodes. In distant lymph nodes of 5 of those 6 patients MDM was found. There was only 1 patient with histologically and MB proved tumor free local lymph nodes, in which histologically proved metastasis in a distant lymph node (LN9) occurred (for more detailed information about this patient see Table 18, patient 25).

Only 12 patients (44,4%) were MB positive (being histologically negative) in the distant lymph nodes. These 12 patients were further included in search of clinical relevance of distant nodal MDM.

Table 5. Patients with histologically and MB proved lymph nodes involvement from the both groups (distribution among regional and distant lymph nodes).

Level of lymph nodes involvement	Histology	MB (K-ras+ CK20)	Any of the methods
Patients without lymph node involvement	29.6% *(8/27)*	29.6% *(8/27)*	11.1% *(3/27)*
Patients with Regional involvement	66.7% *(18/27)*	42.9% *(3/7)* [1]	66.7% *(18/27)* [2]
Patients with Distant involvement	33.3% *(9/27)*	74.1% *(20/27)*	77.8% *(21/27)*
Patients with only Distant involvement	3.7% *(1/27)**	18.5% *(5/27)*	22.2% *(6/27)*

[1] - the index was investigated only in regional lymph nodes of the patients from the 2nd group.
[2]- the regional lymph nodes of the patients of the 1st group were investigated only histologically.
***-** corresponds to patient 25 in Table 14.

3.4 The influence of different morphological parameters on distant nodal MDM appearance in patients with pancreatic cancer.

In order to find the factors influencing the distant nodal MDM appearance, the patients were divided into different groups within certain clinical or morphological parameters. In a group of pN0 patients consisting of 8 persons we found 5 patients (62.5%) with MDM in distant lymph nodes. A group of patients with only regional histologically positive lymph nodes (pN1, according JPS), consisting of 10 persons had 7 patients (70%) with MDM. For another perspective, among 12 pN2 negative patients with MDM, 5 (41.7%) were pN0 patients and 7 (58.3%) – pN1 patients (Table 6).

Table 6. Correlation between distant nodal MDM and histological local lymph node status.

Patients	Number	pN_0 – patients	Patients with only local lymph nodes involvement
All patients	**27**	*8*	*10*
PN2 negative patients with MDM in distant lymph nodes	**12**	*5* (41.7.%)	*7* (58.3%)

Table 7 represents the distribution of distant nodal MDM among patients with histological tumors grade I-II and higher than II; among patients with tumor status 1-2 and 3-4 (according to UICC), and patients with and without histologically detected lymph vessels invasion.

Table 7. Appearance of distant nodal MDM among patients with different morphological parameters.

Patients	Number	Tumor Grade		Lymph vessels invasion		pT	
		G1/G2	> G2	+	-	T_1 / T_2	T_3 / T_4
All patients	**27**	*14* (51.8%)	*13* (48.2%)	*9* (33.3%)	*18* (66.7%)	*5* (18.5%)	*22* (81.5%)
PN2 negative patients with MDM in distant lymph nodes	**12**	6 (50.0%)	6 (50.0%)	*3* (33.3%)	9 (66.7%)	4 (25.0%)	8 (75.0%)

Chi-square test showed no significant connection between any of the morphological factors and MDM appearance in distant lymph nodes: for all the factors $p > 0,27$.

3.5 MDM and metastases spread in distant lymph nodes in patients with adenocarcinoma of the head of the pancreas. Influence on local recurrence of the disease.

The most frequent site of nodal involvement for patient with pancreatic head carcinoma were para-aortal lymph nodes (LN16) – 12 patients (Table 8). Of these, 7 of them were found to have signs of MDM and 5 had metastases. Involvement of the lymph nodes in hepato-duodenal ligament (LN12) occurred in 7 patients (2 and 5 respectively). Also, lymph nodes along superior mesenteric artery (LN 14) were involved in 7 patients (5 and 2 respectively), lymph nodes along celiac trunc (LN9) - in 5 (2 and 3 respectively) and metastasis in lymph nodes along the splenic artery (LN11) in 1 patient. If the molecular marker was positive in a lymph node with metastasis, it was not considered a MDM. Fig. 4 also represents the differences in results when using routine histological and molecular methods.

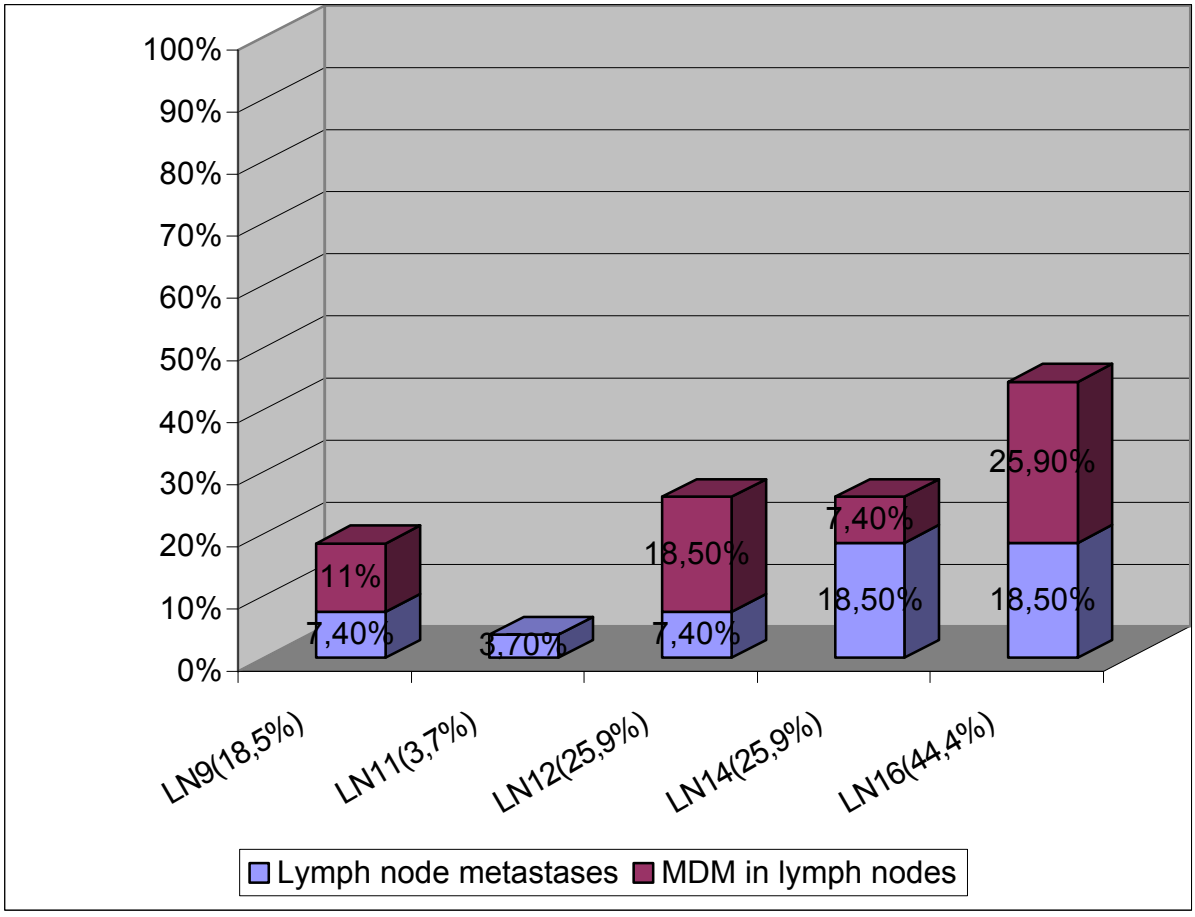

Fig. 4. Distribution of metastases and MDM among distant lymph nodes.

Altogether, there were 18 patients investigated for distant nodal MDM (Table 8) with no distant lymph node metastases.

Table 8. Comparison of progression free survival among patients with distant lymph node metastasis and MDM in different stations.

LN	Distant nodal MDM				Distant lymph node metastases			
	number 18	FT, median (range)	PFS, median (range)	2-year PFS	number 27	FT, median (range)	PFS, median (range)	2-year PFS
9	2/ 11*%*	1; 37	1; 37	1/*50%*	3/ *11%*	6; 11	5; 6	0
11	0/ *0*	-	-	0	1/ *4%*	11	5	0
12	5/ *28%*	20 (1-37)	20 (1-37)	2/*40%*	2/ *7%*	1; 7	1; 7	0
14	2/ *18%*	6; 40	6; 40	1/*50%*	5/ *19%*	9 (1-35)	8 (1-35)	0
16	7/ *39%*	13 (2-40)	13 (8-28)	1/*14%*	5/ *19%*	7 (1-11)	5	0
N0	6 / *33%*	34 (17-39)	26 (7-39)	4 / 67*%*	8 / *30%*	20 (1-40)	20 (1-40)	5 / *63%*

LN – lymph node station, according to JPS, **N** – number of patients with any nodal involvement, **FT**- follow up time, in months. **PFS** – progression free survival, in months. **N0** – patients with out MDM and metastasis respectively.

Among the 7 patients with MDM in LN16, it was combined with MDM in LN12 in one patient.

In 2 patients with MDM in LN14, one had histological metastasis in LN9. Among 5 patients with MDM in LN 12, one had MDM in LN 9, and one in LN16. In the rest of the included patients, MDM occurred only in a single LN station.

The evidence of MDM in LN 16 was associated with the lowest 2-year PFS. The overall survival (OS) of LN16- positive and negative patients would be compared in chapter 3.6.5 using different criteria.

3.6. Distant nodal MDM. The influence on clinical prognosis in patients with adenocarcinoma of the head of the pancreas.

3.6.1. Clinical parameters of the patients with and without the signs of distant nodal MDM. MDM evaluated using both K-ras and CK 20 as molecular markers.

Among the 28 patients with adenocarcinoma of the pancreas, 20 had positive molecular signs of distant lymph nodes involvement. In 8 of those 20 molecular signs were associated with histologically proved distant lymph node metastases. These 8 patients were excluded from further study.

No molecular signs of nodal MDM were observed in 8 of 28 patients. One of these 8 had multiple distant lymph nodes metastases and one had carcinoma of the tail of the pancreas and diffuses liver metastases. Both of these patients were excluded from further investigation.

For the analysis of influence of the distant nodal MDM on clinical outcome, only 18 patients with distant lymph nodes free of histologically proved metastases (pN2-negative) were included. In these patients the overall survival (OS) and progression free survival (PFS) were analyzed. Of 6 patients without MDM the actual 2-year survival and the 2-year PFS were the same 67% (4 patients). The median survival was more then 34 (range, 17-39) months and median PFS was 26 (range, 7-39) months (Table 9, marked strings).

Among 12 patients with nodal MDM (MDM +), 3 died for reasons unrelated to the disease progression (Table 9). The actual 2-year survival was 42% (5 patients), the 2-year PFS was 33% (4 patients), the median survival for this group was 20 (range, 1-40) months and the median PFS – 18 (range, 1-37) months.

The Kaplan-Meier curves for OS in months are represented by Fig. 5.

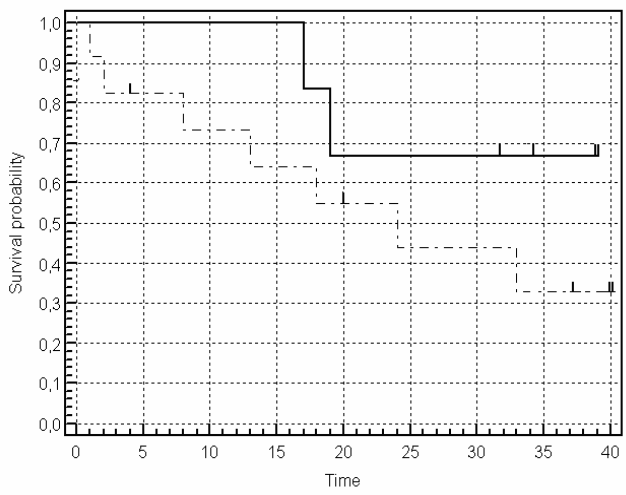

Fig. 5. Comparison of OS (in months) of the patients with and without (solid line) the signs of distant nodal MDM.

The difference in OS was statistically not considerable, p=0.24.

Table 9. pN2-negative patients with and without the signs of distant nodal MDM.

Nr.	pN	K-ras prim tumor	Mark	MDM in LN (JPS)	Li	G	pT	Adjuvant chemo-therapy	Mts / rec..	PFS (months)	OS (months)	Follow up time (months)
1	1	+	C+ras	16	+	3	3	G/U	No		2*	2
2	0	+	C+ras	12	-	2-3	3	G/U	Yes	20		20
5	1	+	C+ras	9, 12	-	1	3	G/U	Yes	37		37
7	0	+	C+ras	16	-	3	1	ESPAC1	No		13**	13
8	1	+	C+ras	16	-	2	3	ESPAC1	Yes	8	8	8
10	1	+	C	16	-	2	3	G/U	Yes	16	24	24
13	1	+	Ras	16	+	2	2	ESPAC1	Yes	28		40
15	1	+	Ras	12		3-4	3	G/U	Yes	33	33	33
17	0	+	C+ras	12, 16	-	2	3	-	Yes	18	18	18
21	0	-	C	14	-	2	3	G/U	No			40
29	1	-	C	16	-	2-3	2	-	No			4
30	0	+	Ras	12	+	2-3	3	-	No		1***	1
3	0	+	-	-		2-3	4	G/U	Yes	26		39
11	0	+	-	-	-	2	3	-	Yes	8	19	19
16	1	+	-	-		3	3	-	Yes	7	17	17
18	1	+	-	-	+	1-2	3	G/U	No			32
22	0	-	-	-	+	2	3	G/U	Yes	39		39
23	1	-	-	-	-	2	3	G/U	Yes	20		34

K-ras prim.tumor – K-ras status f primary tumor; **Mark**. – type of positive marker (**C**- CK20**, ras**-K-ras); **MDM in LN (JPS)** – number of lymph node with microcarcinois, according to JPS; **Li** – lymphatic vessels invasion; **G**- histological grade of the tumor; **Mts/rec** – distant metastases and/or local recurrence, all patients who developed distant metastases also had local recurrence. **OS –** overall survival. **PFS -** Progression Free Survival. **ESPAC1** – 5FU + folinic acid, **G/U** – Gemzar + Ukrain. *****- patients died of renal failure. ******- patient died because of lung cancer. ******* - patient died of respiratory insufficiency.

Table 10 and Table 11 were designed to follow the different periods of observed survival.

Table 10.a. The OS of the patients with and without distant nodal MDM, months.

OS, months		**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**	**30**	**33**	**36**	**39**
Number of patients	**MDM "+"**	**12**	10	9[1]	8	8 *67%*	7	7	5[1]	5 *42%*	4	4	4	3 *25%*	2[1]
	MDM "-"	**6**	6	6	6	4 *67%*	6	5	4	4 67%	4	4	3[1]	2[1] *33%*	2

[1]-1 patient censored. Patients were censored when they were out of the follow up.

Table 10.b. The OS of the patients with and without distant nodal MDM, percentiles.

Percentile		100th	84th	75th	67th	50th *median*	33rd	25th	17th
Months of survival	**MDM "+"**	1	4[1]	8	13	20[1]	33	37[1]	40[2]
	MDM "-"	17	32	-	32	34	-	39	39

[1] - 1 patient was censored.
[2] - 2 patients censored. Patients were censored when they were out of the follow up

Table 11.a. The PFS rate of survival rate of the patients with and without distant nodal MDM, months.

PFS rate, months		0	3	6	9	12	15	18	21	24	27	30	33	36	39
Number of patients	**MDM "+"**	**12**	10[2]	9[1]	8	8 *67%*	7[1]	6	4	4 *33%*	4	3	3	2 *17%*	1
	MDM "-"	**6**	6	6	4	4 *67%*	4	4	3	3 *50%*	2	2	1[1]	1 *17%*	1

[1] - 1 patient was censored.
[2] - 2 patients censored. Patients were censored when they were out of the follow up or died with no signs of disease progression.

Table 11.b. The PFS rate of the patients with and without distant nodal MDM, percentiles.

Percentile		100th	84th	75th	67th	50th *median*	33rd	25th	17th
Months of survival	**MDM "+"**	1	4[2]	8[1]	13[1]	18	28	37	37
	MDM "-"	7	8	-	20	26	32[1]	-	39

[1] - 1 patient was censored.
[2] - 2 patients censored. Patients were censored when they were out of the follow up or died with no signs of disease progression.

3.6.2. Survival rates in patients with and without the signs of distant nodal MDM. MDM evaluated on the basis of CK 20 expression as a marker.

In order to evaluate the possible influence of every factor (CK20 or K-ras) separately, a group of patients with only CK 20 as a positive marker for distant nodal MDM (CK 20^{+}) was compared to those with CK 20 negative lymph nodes (CK 20^{-}) (Fig. 6).

Among 9 patients with $CK20^{+}$ distant lymph nodes, the actual 2-year survival rate was 33% (3 patients), the 2-year PFS rate was 22% (2 patients), the median survival was 18 (range, 2-40) months and the median PFS was 16 (range, 2-37) months.

Among 9 $CK20^{-}$ patients, the actual two-year survival rate was 67% (6 patients), the 2-year PFS rate was 56% (5 patients), the median survival was 33 (range, 1-40) months and the median PFS was 26 (range, 1-39) months.

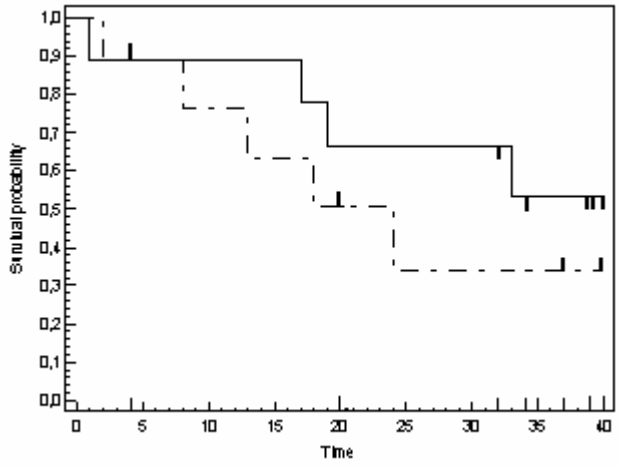

Fig. 6. Comparison of OS (in months) of the patients with and without $CK20^{+}$ (solid line) distant nodal MDM.

The difference in OS of the patients with and without CK 20-proved distant nodal MDM did not reach statistical significance, p=0.38 (Fig. 6). Table 12.a and 14.b were designed to follow the different periods of PFS. Patients were censored when they were out of the follow up or died with no signs of disease progression.

Table 12.a. The PFS rate of the patients with and without $CK20^{+}$ distant nodal MDM, months.

PFS rate, months		0	3	6	9	12	15	18	21	24	27	30	33	36	39
Number of patients	CK20 "+"	9	8[1]	7[1]	6	6	51	4	2[1]	2	2	2	2	2	1
	CK20 "-"	9	8[1]	8	6	6	6	6	5	5 *56%*	4	3	2[1]	1	1

[1] - 1 patient censored.

Table 12.b. The PFS rate of the patients with and without $CK20^{+}$ distant nodal MDM, percentiles.

Percentile		100th	89th	78th	67th	56th	50th *median*	44th	33rd	22nd	11th
Months of survival	**CK20 "+"**	21	41	8	131	16	16	18	20	37	40
	CK20 "-"	11	7	8	20	26	26	28	32	33	39

[1]- 1 patient censored.

3.6.3. Survival rates in patients with K-ras positive primary tumor with and without the signs of distant nodal MDM. MDM evaluated on the basis of K-ras mutation as a marker.

3.6.3.1. K-ras mutation in primary tumor. Influence on progression free and OS.

In order to evaluate the influence of K-ras mutation in primary tumors on OS and PFS, 27 patients were divided into 2 groups. The first group consisted of 5 patients with K-ras negative primary tumors. The second group consisted of 22 patients with K-ras positive primary tumors. In statistical analysis Log rank test showed essential difference in OS between these two groups, p=0.04 (Fig. 7).

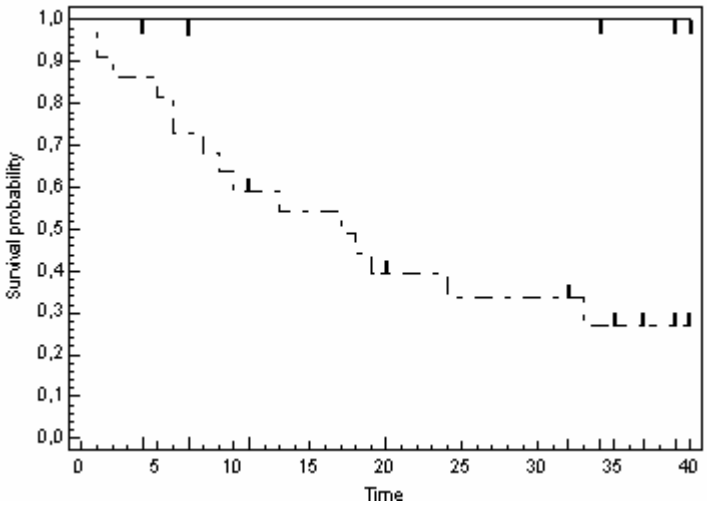

Fig. 7. The OS (in months) of the patients with and without (solid line) K-ras mutation in primary tumor.

In K-ras positive patients the actual 2-year survival was 32% (7patients), the 2-year PFS was 27% (6 patients), the median survival – 17 (range, 1-40) months and the median PFS was 8 (range, 1-37) months. Whereas, all 5 K-ras negative patients were alive by the time of the latest check, the actual 2-year survival for them was 60% (3 patients), the 2-year PFS was 40% (2 patients) the median survival – 34 (range, 4-40) months and the median PFS was 20 (range, 4-39).

Further search of influence of the MDM -factor on clinical outcomes was carried out only in K-ras positive groups.

3.6.3.2. Influence of K-ras proved MDM on survival rates.

When 14 patients with K-ras positive primary tumor and without metastases distant (N2) lymph nodes were analysed, they were divided into two groups: those with and those without distant nodal MDM. In these only K-ras was considered as the marker (Fig. 8). Among 5 MDM -negative patients the actual 2-year survival was 60% (3 patients), the 2-year PFS rate was 40% (2 patients), the median survival was 24 (range, 17-39) months and the median PFS was 16 (range, 7-32) months.

Among 9 MDM -positive patients, the actual 2-year survival was 33% (3 patients), the 2-year PFS rate was 33% (3 patients), the median survival was 18 (range, 1-40) months and the median PFS was 18 (range, 1-37) months.

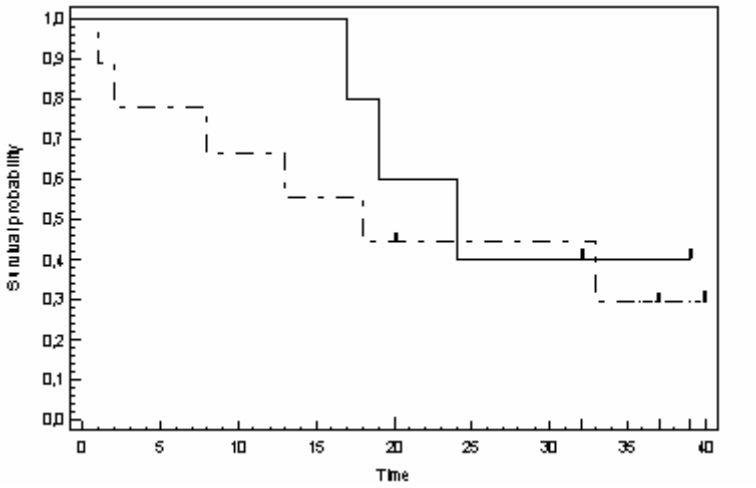

Fig. 8. Comparison of OS (in months) of the patients with K-ras positive primary tumor with and without K-ras-proved (solid line) distant nodal MDM.

The difference in the patients with and without K-ras-proved distant nodal MDM was not statistically considerable, p=0,58 (Fig. 8). Table 13a and 15b were designed to follow the different periods of PFS. Patients were censored when they were out of the follow up or died with no signs of disease progression.

Table 13. a. The PFS rate of the patients with K-ras positive primary tumor with and without distant nodal MDM, months.

PFS rate, months		0	3	6	9	12	15	18	21	24	27	30	33	36	39
Number of patients	MDM "+"	9	7[2]	7	6	6 *67%*	5	5	3[1]	3 *33%*	3	3	3	2[1] *22%*	1[1]
	MDM "-"	5	5	5	3	3 *60%*	3	2	2	2 *40%*	1	1[1]	-	-	-

[1] - 1 patient censored.
[2] - 2 patients censored.

Table 13.b. The PFS rate of the patients with K-ras positive primary tumor with and without distant nodal MDM, percentiles.

Percentile		100th	89th	80th	78th	67th	60th	56th	50th	44th	40th	33rd	22nd	20th	11th
Months of survival	MDM "+"	1[1]	2[1]	-	8	13[1]	-	18	18	20[1]	-	28	33	-	37
	MDM "-"	7	-	8	-	-	16	-	16	-	26	-	-	32[1]	-

[1] - 1 patient censored.

3.6.4. Survival rates in patients with K-ras positive primary tumor with and without the signs of distant nodal MDM. MDM considered when K-ras and CK20 markers were concordant positive in the same lymph node.

Afterwards the groups of patients with K-ras positive primary tumors were rearranged. Those were considered as MDM positive, who had MDM proved by concordance of both positive molecular markers in the same tissue sample (lymph node). Among the latter were patients 1, 2, 6, 7, and 16 (Table 9). In this group no 2-year survivors were observed; the median survival and the median PFS were the same 13 (range, 2-20) months.

In the MDM negative group were patients 3, 10, 15, 17; they had no signs of MDM at all. The 2-year actual survival rate was 50% (patients), the 2-year PFS rate was also 50% (2 patients), the median survival for this group was 32 (range, 17-39) months and the median PFS 26 (range, 7-32) months (Table 14). However, there was also no essential differences in survival rates, p=0,11 (Fig. 9).

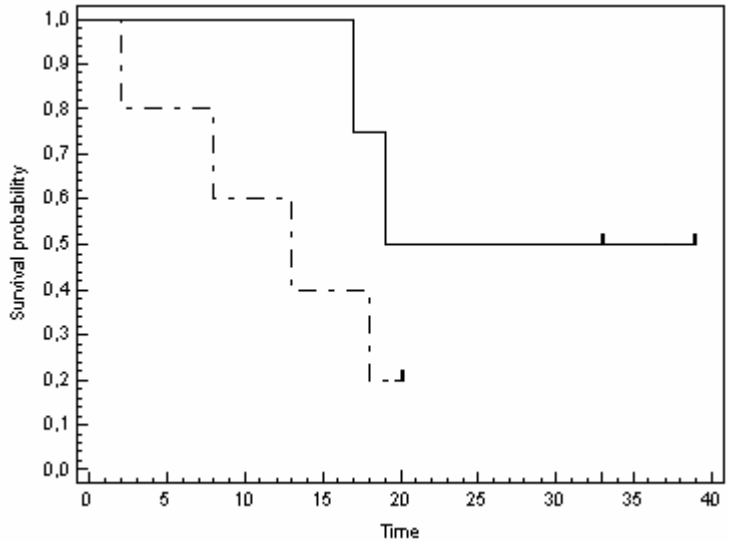

Fig. 9. Comparison of OS (in months) of the patients with K-ras positive primary tumor with and without (solid line) distant nodal MDM. MDM was proved by concordant positive K-ras and CK 20 markers.

Table 14.a and 16.b were designed to follow the different periods of PFS. Patients were censored when they were out of the follow up or died with no signs of disease progression.

Table 14.a. The PFS rate of the patients, with K-ras positive primary tumor, with and without distant nodal MDM evaluated by concordant results, in months.

PFS rate, months		0	3	6	9	12	15	18	21	24	27	30	33	36	39
Number of patients	MDM "+"	5	4[1]	4	3	3	2[1]	1	0	0	0	0	0	0	0
	MDM "-"	4	4	4	2	2	2	2	2	2 *50%*	1	1	0[1]	-	-

[1] - 1 patient censored.

Table 14.b. The PFS rate of the patients with K-ras positive primary tumor with and without distant nodal MDM evaluated by concordant results, in percentiles.

Percentile		100th	80th	75th	60th	50th *median*	40th	25th	20th
Months of survival	**MDM "+"**	2[1]	8	-	13[1]	13	18		20[1]
	MDM "-"	7	-	8	-	26	-	32	-

[1] - 1 patient censored.

3.6.5. Survival rates in patients with K-ras positive primary tumor with and without MDM I LN 16. MDM considered as concordant positive K-ras and CK20 marker in the same lymph node.

The group of patients with MDM in LN 16 proved by concordant positive K-ras and CK20 markers (LN16-MDM -positive) was compared to patients without any sign of MDM (MDM -negative) and with the rest of the pN2 negative patients (LN16-MDM -negative) (Fig. 8.a, 9b). The LN16 positive group consisted of 4 patients (Table 9, N. 1, 6, 7, 16). There were no 2-year survivors among them; the median overall and progression free time were both equal to 13 (range, 2-18) months (patient 1 died in the 2nd month postoperatively because of renal insufficiency).

MDM -negative group was the same as in the previous comparison and also included 4 patients (Table 9, N. 3, 10, 15, 17).

The LN16-MDM -negative patients, in whom the concordance of both markers was not identified in LN 16 consisted of 10 patients (MDM -negative group included). The actual 2-year survival for the group of LN16 negative patients was 60% (6 patients), the 2-year PFS – 50% (5 patients), the median survival - 32 (range, 1-40) months and the median PFS – 26 (range, 1-33) months (Table 15).

In a comparison of OS between LN16-MDM -positive and MDM -negative patients by log rank test the difference was statistically considerable (Fig. 10.a).

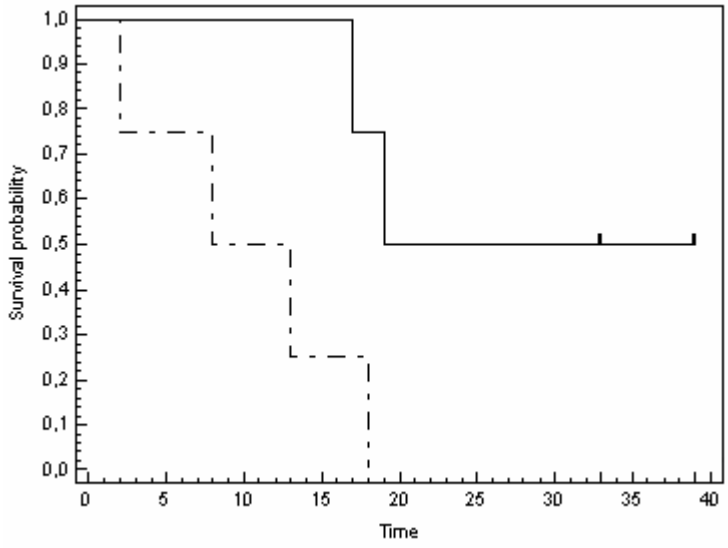

Fig. 10.a. Comparison of OS (in months) of LN16-MDM -positive and MDM -negative (solid line) patients. MDM was proved by concordant positive K-ras and CK 20 markers, p=0.03.

When the OS in LN-MDM -16 positive patients and in 6 patients in whom the MDM was not proved by concordance of both markers in LN 16 (MDM negative group not included) were compared by log rank test, the difference was also essential, p=0.02. The latter group of 6 patients was compared to the MDM negative group and the p-value formed 0.86, which proves that there is no essential difference in OS in these two groups (data not shown).

When the OS of the LN16-MDM -positive group was compared to the OS of the LN16-MDM -negative group consisting of 10 patients (MDM negative included), the difference was essential (Fig. 10.b).

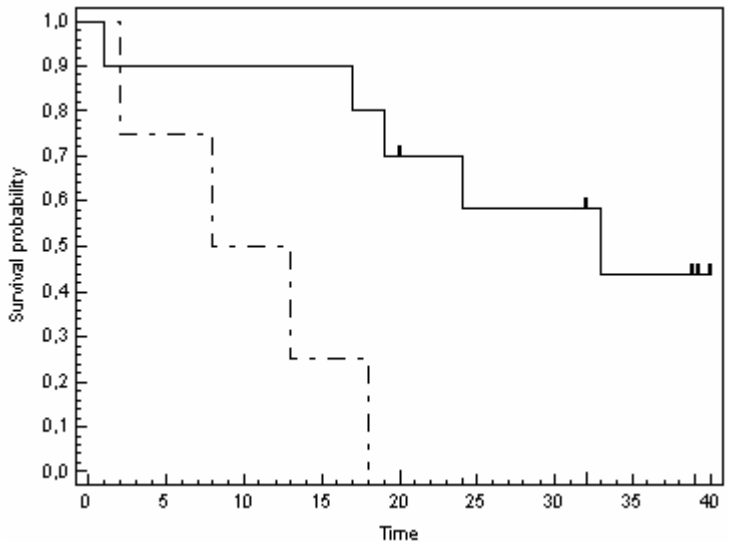

Fig. 10.b. Comparison of OS (in months) of the LN16-MDM -positive patients and LN16-MDM -negative patients (solid line). MDM was proved by concordant positive K-ras and CK 20 markers, p=0.005.

Table 15.a and 17.b were designed to follow the different periods of PFS. Patients were censored when they were out of the follow up or died with no signs of disease progression.

Table 15.a. The PFS rate of the patients, with K-ras positive primary tumor, with and without distant nodal MDM in LN 16 evaluated by concordant results, in months.

PFS rate, months		**0**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**	**27**	**30**	**33**	**36**	**39**
Number of patients	**LN16 "+"**	**4**	3[1]	3	2	2	1[1]	1	0	0	0	0	0	0	0
	LN16 "-"	**10**	9[1]	9	7	7	7	6	5	5 *50%*	4	3	2[1]	1	1

[1] - 1 patient censored.

Table 15.b. The PFS rate of the patients with K-ras positive primary tumor with and without distant nodal MDM in LN 16 evaluated by concordant results, in percentiles.

Percentile		100th	90th	80th	75th	70th	60th	50th	40th	30th	25th	20th	10th
Months of survival	LN16 "+"	2[1]	-	-	8	-	-	13[1]	-	13	-	18	-
	LN16 "-"	1[1]	7	8	-	16	20	26	28	32[1]	-	33	37[1]

[1] - 1 patient censored.

The results of the PFS comparison for these groups of patients are represented in Table 21.

3.7 Another factors influencing the clinical prognosis in patients with adenocarcinoma of the panceatic head.

3.7.1 Survival in patients with and without lymph node metastases

The OS and PFS rates were also analyzed in the following groups of patients: 8 patients "pN0" and 19 patients "pN1, pN2 positive". The pN1, pN2 positive group corresponds to pN1 group according to UICC classification. The difference in OS was not considerable (p=0.44) when evaluated by Log rank test (Fig. 11.).

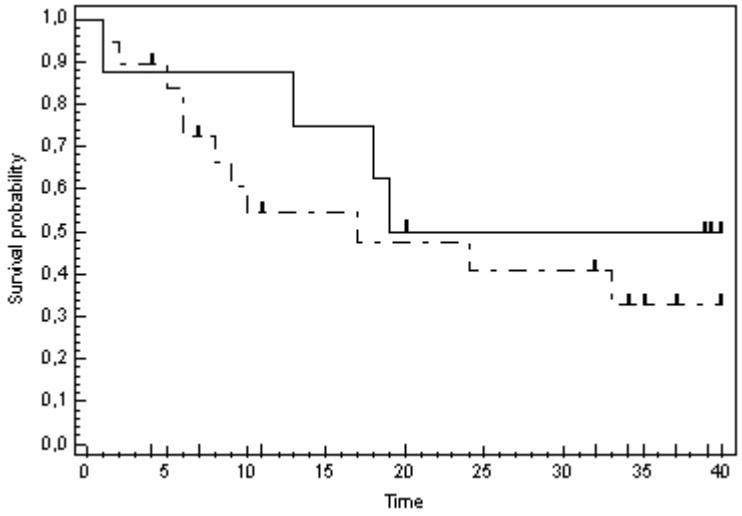

Fig. 11. The OS (in months) for pN0 patients (solid line) and pN1, pN2 positive patients.

In pN0 group the actual 2-year survival was 38% (3 patients), the 2-year PFS was 25% (2 patients), the median survival – 20 (range, 1-40) months and the median PFS was 20 (range, 1-40) months. The actual 2-year survival for the pN1, pN2 positive patients was 37% (7 patients), the 2-year PFS was 21% (4 patients), the median survival – 10(range, 1-40) months and the median PFS was 8 (range, 1-37).

3.7.2. Survival in patients with and without histologically proved distant nodal metastases

Comparing the OS in patients groups with (9 patients) and without (18 patients) distant lymph node metastases ("$pN2^{+}$" and "$pN2^{-}$" resp.), a clear tendency for higher survival rates in the $pN2^{-}$ group appeared; but the Log rank test showed p-value equal to 0.0545 (Fig. 12).

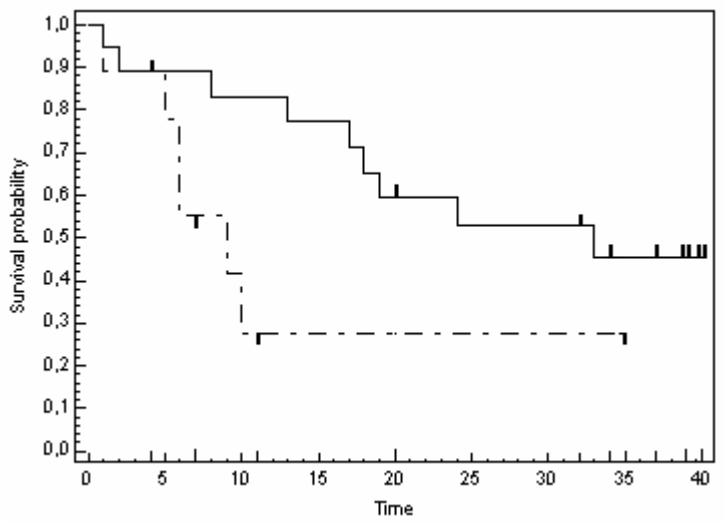

Fig. 12. Comparison of OS (in months) in pN2 negative (solid line) and pN2 positive patients, p=0.0545.

For pN2 negative and pN2 positive patients respectively, the actual 2-year survival rates were 50% (9 patients) and 11% (1 patient); the 2-year PFS was 39% (7 patients) and 11% (1patient); the median survival rate was – 24 (range, 1-40) months and 7 (range,1-35) months and the median PFS rate - 20 (range, 7-40) months and 6 (range, 5-35) months.

3.8 The influence of different adjuvant chemotherapy protocols on survival rates of R0 resected patients with adenocarcinoma of the pancreas.

In the postoperative period 23 of 27 patients received adjuvant chemotherapy (CT) according to two different protocols: ESPAC-1 (4 patients) and Gemcytabine+Ukrain (G/U) (15 patients). The OS of patients treated with G/U protocol, ESPAC protocol and those, who received no CT (8 patients) were compared among themselves (Fig. 13. a-c).

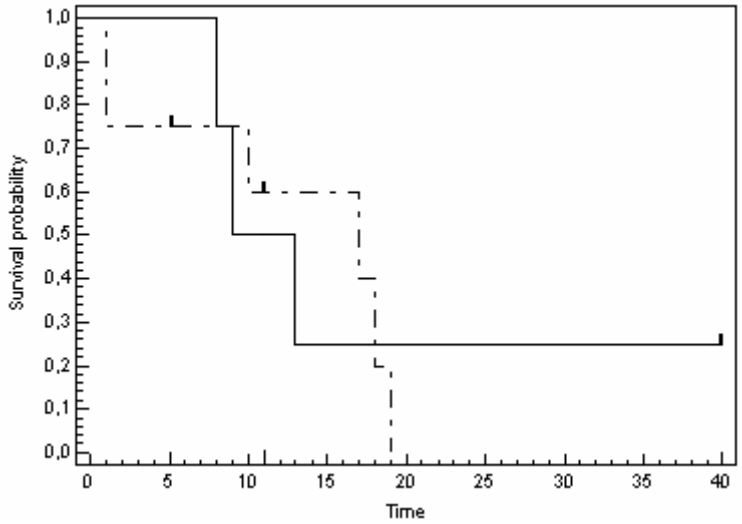

Fig. 13. a The OS (in months): patients treated with ESPAC-1 (solid line), versus patients without adjuvant CT, p=0.87.

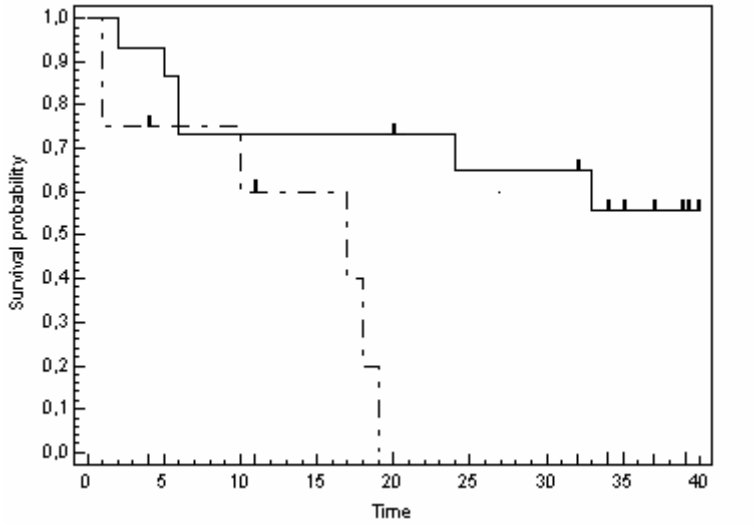

Fig. 13. b The OS (in months): patients treated with G/U protocol(solid line), versus patients without adjuvant CT, p=0.01

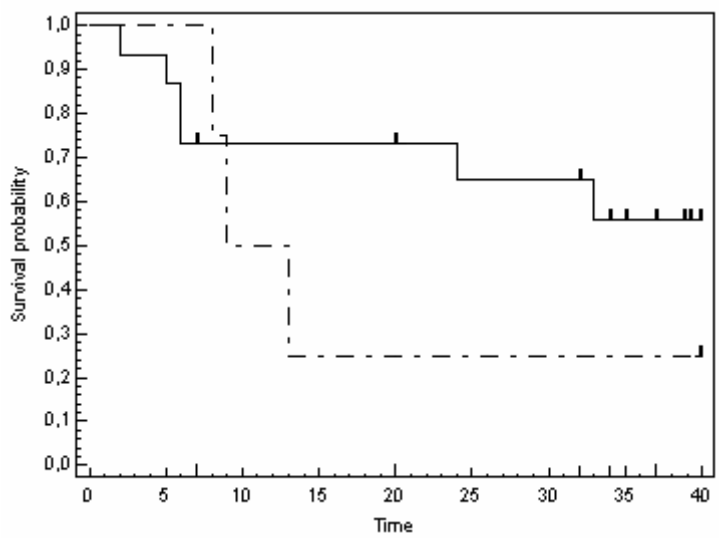

Fig. 13. c. The OS (in months): patients treated with G/U protocol (solid line), versus ESPAC-1, p=0.32

The median survival rates for these patients' groups are represented in Table 16.

Table 16. Survival rates of patients with different adjuvant treatment protocols, in months.

CT protocol	Number of patients	Median survival	Median PFS	Actual 2-year survival	2-year PFS
No CT	**8**	10 (range, 1-19)	7 (range, 4-18)	0	0
ESPAC-1	**4**	13 (range, 8-40)	13 (range, 8-28)	1 (25%)	1 (25%)
G/U	**15**	32(range, 2-40)	20 (range, 5-40)	9 (60%)	7 (47%)

Table 17 represents the distribution among three groups of such parameters as tumor histological grade and anatomical extent, as proven true prognostic factors according to the UICC (Hermanek). Factors such as K-ras – status of primary tumor and pN-status are also included for prognostic relevance for the patients in this study.

Table 17. Structure of groups of patients with different adjuvant chemotherapy regimen.

	Total	=G2	>G2	T1-T2	T3-T4	N0	N1	N2	ras"+"	ras"-"
No CT	**8**	4 50%	4 50%	2 25%	6 75%	4 50%	2 50%	2 25%	7 88%	1 12%
ESPAC	**4**	2 50%	2 50%	2 50%	2 50%	1 25%	1 25%	2 50%	4 100%	0
G/U	**15**	9 60%	6 40%	1 7%	14 93%	4 27%	6 40%	5 33%	11 73%	4 27%

N-status according to JPS

From the group of CT untreated patients, 2 were alive at the time of the latest check. The follow up times for these patients were 11 and 4 months, respectively. The first one has developed recurrence in the 5th month after surgery.

Of the 4 patients from the ESPAC group, 1 patient was alive at the time of the latest check (follow up time 40 months); he has developed recurrence in the 28th month after surgery.

Of the 15 patients from the G/U group, 8 patients were alive at the time of the latest check. Of these, 4 patients have no progression signs (follow up time 7, 32, 35 and 40 months respectively); 4 patients with follow-up of 39, 37, 39 and 34 months have developed recurrence at 26, 37, 39 and 20 months respectively. One of them, (patient 21), had local recurrence in the pancreas bed and one macroscopic liver metastasis. She has undergone relaparatomy and today (12 months after the second operation) has no signs of progression.

One patient, who had disease progression diagnosed in the 20th month after surgery is no longer available for follow up.

Table 18. Clinical-morphological parameters of 28 patients with pancreatic cancer. Update - 14.10.2003.

Nr.	Sex	Age	St	pN	K-ras prim tumor	Metastases in distant LN (JPS)	MDM LN (JPS)	Li	Pi	Vi	Ppi	G	pT	OP procedure	Adjuvant therapy.	Mts / rec..	PFS, months	OS, months
1	M	74	III	1	+		16	+			+	3	3	PpWhipple	G/U	No		2[1]
2	F	68	II	0	+		13.12	-			+	2-3	3	ppWhipple	G/U	Yes	20	20*
3	M	64	IV	0	+		-			+		2	4	ppWhipple	G/U	Yes	26	39+
4	F	74	III	1	+		9.12.17	-	+			1	3	ppWhipple	G/U	Yea	37	37+
5	F	75	III	1	+	14.16	14.16	-	+			2-3	3	ppWhipple	No	Yes	8	10
6	F	61	I	0	+		16	-	-			3	1	ppWhipple	ESPAC 1	No		13[2]
7	F	71	III	1	+		16	-	-			2	3	ppWhipple	ESPAC 1	Yes	8	8
8	M	45	III	1	+	14	14	-			+	2-3	3	ppWhipple	ESPAC 1	Yes	9	9
9	M	56	III	1	+		16	-	-			2	3	ppWhipple	G/U	Yes	16	24
10	M	62	II	0	+		-	-			+	2	3	ppWhipple	No	Yes	8	19
11	F	73	III	1	+	11.14.16	14.16	+	+			2	3	ppWhipple	G/U	Yes	5	5
12	M	63	III	1	+		16	+	-			2	2	ppWhipple	ESPAC 1	Yes	28	40+
13	F	52	III	1	+	14	14	-	+			3	3	ppWhipple	G/U	No		35+
14	M	64	III	1	+		12		+	+		3-4	3	ppWhipple	G/U	Yes	33	33
15	F	73	III	1	+		-				+	3	3	ppWhipple	No	Yes	7	17
16	F	70	II	0	+		12.16	-			+	2	3	ppWhipple	No	Yes	18	18
17	M	58	III	1	+		-	+	+			1-2	3	ppWhipple	G/U	No		32+
18	M	69	III	1	+	12.14.16	9.12.14.16	+	+			2-3	3	ppWhipple	No	No		1[3]
19	F	72	II	0	-		14	-	+			2	3	ppWhipple	G/U	No		40+
20	M	65	II	0	-		-	+			+	2	3	ppWhipple	G/U	Yes	39	39+
21	F	29	III	1	-		-	-				2	3	ppWhipple	G/U	Yes	20	34+
22	M	45	III	1	+	8;9;11;16	8;9;11;13;16	+	+	-		2	2	ppWhipple	DC	Yes	5	11+
23	F	58	IV	1	+	8;9;	9;13	+	-	+		3	4	ppWhipple	G/U/DC	Yes	6	6
24	F	31	III	1	-	8;12;16		-	-	-		3	2	ppWhipple	G/U/DC	No	7	7+
25	F	59	III	1	+	9	14	-	-	-		2	3	ppWhipple	G/U/DC	Yes	6	6
26	F	74	IV	1	-			-	-	-		2	2	D.pncrtmyR2	G/U/DC	Yes'		4+
27	M	47	III	1	-		16	-	-	+		2-3	2	ppWhipple	DC	No		4+
28	M	65	II	0	+		12	+	-	-		2-3	3	T. pncrtmy	No	No		1[4]

St. –tumor stage (UICC)**, K-ras prim.tumor** – K-ras status f primary tumor; **Mark**. – type of positive marker (**C**- CK20**, ras**- K-ras); **LN (JPS)** – number of lymph node, according to JPS; **Li** – lymphatic vessels invasion; **Pi** – perineural invasion, **Vi** – blood vessels invasion, **Ppi** – peripancreatic invasion,**G**- histological grade of the tumor; **D** and **T pncrtmy** – distal and total pancreatectomy resp.**G/U** – Gemzar + Ukrain, **ESPAC-1**- 5FU + folinic acid; **Mts/rec** – distant metastases and/or local recurrence, all patients who developed distant metastases also had local recurrence.

1 - patients died of renal failure

2 - patient died because of lung cancer

3– patient died of heart attack

4 - patient died of respiratory insufficiency

+ - patients are alive

*****- patient is lost for follow up

Patient **28** had cancer of the tail of the pancreas and diffuse liver metastases and was excluded when survival rates were evaluated.

3.9 Statistics of the survival rates in groups with different investigated parameters in patients with adenocarcinoma of the pancreas.

The exploratory p-values for comparison of survival rates for patients classified according to investigated parameter are represented in Table 19.

Table 19. Exploratory P-value in comparison of different clinical, morphological and molecular parameters influencing survival in 27 patients with adenocarcinoma of the pancreatic head.

Parameter of comparison	Number of patients	Log rank test for OS comparison, p-value	Median survival, months (range)	Median PFS, months (range)	2-year actual survival (patients)	2-year PFS (patients)
Samples taken from population of K-ras positive-pN2 negative patients, n = 14						
MDM (K-ras)"+"	**9**	P = 0.58	18 (1-40)	18 (1-37)	33% (3)	33% (3)
MDM (K-ras)"-"	**5**		24 (17-39)	16 (7-32)	60% (3)	40% (2)
MDM '+' [1]	**5**	P = 0.11	13 (2-20)	13 (2-20)	0	0
MDM '-'	**4**		32 (17-39)	26 (7-32)	50% (2)	50% (2)
MDM in LN16 "+" [1]	**4**	P<0.005	13 (2-18)	13 (2-18)	0	0
MDM in LN16 " - "	**10**		32 (1-40)	26 (1-33)	60% (6)	50% (5)
Samples taken from population of pN2 negative patients, n =18						
MDM "+" [2]	**12**	P = 0.24	20 (1-40)	18 (1-37)	42% (5)	33% (4)
MDM "-"	**6**		>34 (17-39)	26 (7-39)	67% (4)	67% (4)
MDM in LN 16 [2]	**7**	P = 0.08	18 (2-40)	13 (8-28)	28% (2)	14% (4)
MDM "-"	**6**		>34 (17-39)	26 (7-39)	67% (4)	60% (4)
MDM (CK 20) "+"	**9**	P =0.38	18 (2-40)	16 (2-37)	33% (3)	22% (2)
MDM (CK 20)" –"	**9**		33 (1-40)	26 (1-39)	67% (6)	56% (6)
Samples taken from the general population of patients, n= 27						
Prim. tumor K-ras'-'	**5**	P =0.04*	34 (4-40)	20 (4-39)	60% (3)	40% (2)
Prim. tumor K-ras'+'	**22**		17 (1-40)	8 (1-37)	32% (7)	27% (6)
pN0	**8**	P = 0.44	20 (1-40)	20 (1-40)	38% (3)	25% (2)
pN1, pN2 "+"	**19**		10 (1-40)	8 (1-37)	37% (7)	21% (4)
pN2 "+"	**9**	P = 0.0545	7 (1-35)	6 (5-35)	11% (1)	11% (1)
pN2 "-"	**18**		24 (1-40)	20 (7-40)	50% (9)	39% (7)
Adjuvant CT protocol:						
No CT versus ESPAC-1	**8** / **4**	P = 0.87	10 (1-19) / 13 (8-40)	7 (4-18) / 13 (8-28)	0 / 25% (1)	0 / 25% (1)
No CT versus Gemzar + Ukrain	**8** / **15**	P = 0.01*	10 (1-19) / 32 (2-40)	7 (4-18) / 20 (5-40)	0 / 60% (9)	0 / 47% (7)

[1] - evaluated by concordant positive K-ras and ? K 20 marker
[2] - evaluated by at least one positive marker
Prim. –primary

In summary the factors that influenced the clinical outcome of the given population of patients are as follows :

1. The MDM in LN 16 (proved by concordant positive K-ras and CK 20 markers) was associated with lower OS in patients with K-ras positive primary tumor and without metastatic lesions of distant lymph nodes. the OS was evaluated by Kaplan-Meier method and compared by log rank test. In these

patients with MDM in LN 16 (proved by at least one positive marker) the tendency for decreased OS was revealed.

2. The group of patients without metastases in distant lymph nodes (N2"-") tended to have a higher OS and had considerably higher median PFS than N2"+"patients

3. Adjuvant treatment: patients, treated according to "Gemzar + Ukrain" or ESPAC 1 protocol had higher median PFS rate than patients without adjuvant CT. Patients treated by Gemzar and Ukrain also showed higher OS, median survival and 2-year actual survival than untreated patients, what was proved by two statistical methods.

4. The presence of K-ras mutation in the primary tumor correlated with lower OS.

4. DISCUSSION

Despite the considerable progress in our understanding of the pathogenesis and the novel approaches in treatment of pancreatic cancer, the outcome of the disease is still dismal. A majority of patients still die within one year of diagnosis. The only chance for attaining a long-term survival for patients with pancreatic head cancer remains a "curative" resection. The standard method of the resection today is the Whipple procedure which promises up to 33% 5-year survival rates [28] in UICC stage I patients [29].

Of the several independent prognostic factors for long-term survival, the most important is tumor free resection margins (R0 according to UICC) [9, 30, 31]. Also meaningful to prognosis is tumor size and tumor's histological grade [32], although this correlation is not always seen [33]. Recent reports underline the importance of intra- and peri-operative blood transfusions [34]. Lymphovascular, perineural, retroperitoneal and blood vessel invasion also have a considerable negative influence on postoperative survival [35-37]. Kayahara [38] emphasizes the importance of discrimination between intrapancreatic perineural invasion and extrapancreatic neural plexus invasion. He insists on the necessity for mesenteric, coeliac and hepatic plexus dissection in order to perform true R0 resection.

Another significant factor for prognosis is regional lymph node involvement [13, 14, 36, 39]. Although some researchers have failed to prove this in their studies [40], the majority of investigations show that involvement of regional and especially distant lymph nodes is a strong negative prognostic factor in terms of progression free survival and in overall survival (OS) [33, 34, 41, 42]. No connection has been established between the size of the primary tumor and the nodal involvement [40].

The frequency of regional nodal MDM in patients with pancreatic head carcinoma varies from 26% to 73% [23, 24, 43]. More than 40% of the patients develop local-regional recurrence within 6 months and 60-80% do so within 12 months of surgical treatment [11, 12, 13, 44). The most common sites of recurrence are the pancreatic bed, the peritoneum and the liver. The recurrences are seen in some pathohistologically node-negative cases shortly after curative resection. Early lymphatic spread is assumed to be one of the main reasons for this [45]. Obviously routine histological examination with hematoxylin-eosin staining fails to detect cancer involvement in some lymph nodes. That leads to underscoring of the patient's nodal status and error in clinical prognosis evaluation. Gusterson [46] and later Dowlatshahi [47] using serial sectioning of lymph nodes (HE staining and routine histopathologic examination of an extensive number of consecutive sections) showed that the number of positive lymph nodes could be increased in 20%-30% of the specimens. This fact allows for the application of more sensitive methods for nodal status evaluation. Immunohistochemistry and PCR-based techniques are widely performed today for this purpose.

At this point, it would be useful to clarify some peculiarities in the terminology for describing the investigated phenomenon. A lymph node metastasis can be diagnosed when arrest of tumor cell, extravasation, stromal reaction and proliferation occur within specific lymphatic tissue. According to the definition, "micrometastasis" is considered when the focus is less then 0.2 cm [48]. In case of detection of molecular or morphological evidence of tumor cells present in a histologically negative lymph node, Hermaneck proposes to name this phenomenon "microinvolvement" or "microcarcinosis" [31]. We name this phenomenon molecular detected metastases (MDM).

Immunostaining was found to be a sensitive method for detecting a wide range of tumor cells expressing different antigens. The antigens frequently associated with malignancy such as CEA and S 100 for colorectal cancer and melanoma respectively [49,50] and epithelium-specific antigens such as cytokeratins and Ber-Ep 4 are recognized as effective markers for MDM and even tend to have clinical prognostic relevance [24].

Moll et all [51]using immunohistochemistry showed that 18 of 40 cases (40%) of pancreatic carcinoma were positive for CK20 protein expression.

However, there is another sensitive molecular technique for detection of the specific antigens on m-RNA level – RT PCR. That technique allows for the investigation of larger tissue samples. Yamamoto et al. [52] claims that immunohistochemistry has lower false positive rates than non morphological methods, such as flow cytometry or PCR. However, the RT PCR for CK20 detection as a marker for the spread of tumor cells into blood, bone marrow and lymph nodes in gastrointestinal neoplasias, is commonly used [53-56]. Wyld et al. [57] (1998) has determined a frequency of 89% (8 of 9 cases) of CK 20 m-RNA expression in colorectal carcinoma. Chausovsky et al. has investigated blood samples from patients with spread pancreatic cancer and determined that14 of 22 patients (64%) had CK 20 expression in peripheral blood, while 22 patients without malignant disease had no signs of CK 20 expression in their blood [58]. Wildi (1999) in his study demonstrated a 3.2 fold increase in CK 20 m-RNA in 11 of 24 (46%) samples of pancreatic cancer. This allowed Wildi to conclude, that CK 20 can serve as an excellent marker for metastases detection in pancreatic cancer [59].

Apart from detecting the expression of some genes associated with malignancies, PCR techniques are also used in order to identify some genomic mutations in tumor suppressors and protooncogenes such as P53 and K-ras in pancreas cancer patients [43]. Ando [22] have focused on detection of K-ras mutation for MDM in para-aortal lymph nodes of patients with pancreatic adenocarcinoma and have demonstrated the effectiveness of the PCR method. Likewise, Tamagawa and Yamada showed its clinical value in terms of prognosis [26, 27]. The first report about c-Ki-ras gene activation in lymph node metastasis of human pancreatic cancer was published by Prassolov et al. in 1985 [60]. Mutations in codon 12, 13, or 61 of ras genes convert them into active oncogenes [61]. In 1988 Almoguera et al. revealed that the mutation in codon 12 of K-ras genes occurs in about 90% of the cases of exocrine pancreatic cancer [62]. Similar results were obtained later by Hruban et al. who examined 450 specimens of pancreatic adenocarcinoma and reported that the point mutation at K-ras codon 12 was observed in 85% of the cases [63]. In 1991 Kahn et al. proposed a relatively simple and sensitive method for detection of point mutation in K-ras codon 12 - RFLP-based enriched PCR [64]. Kahn's method was used in this study.

In this study both methods RFLP/PCR for genomic K-ras codon 12 mutation and RT PCR for CK 20 expression were used to increase the sensitivity and specificity of detection of nodal MDM. A total of 305 lymph nodes from 28 patients were tested for the presence of K-ras mutation and 237 of those were tested for CK20 expression. In the 305 nodes tested, 249 belonged to twenty-two K-ras positive patients (patients that had K-ras codon 12 mutation in the primary tumor) and 133 of those were also tested for CK 20 expression. For K-ras positive patients the concordance of results of molecular and histological examination was: for "K-ras" method - 89.7%, for "CK 20" - 38.5% , for combination of molecular methods - 96.65%. The evidence of MDM was detected in 25 of 220 (11.4%) in histologically negative lymph nodes by K-ras method, 21 of 120 (17.5%) by CK 20 method and 37 of 220 (16.8%) by combination of molecular methods. In 2 of 29 histologically positive lymph nodes neither signs of K-ras mutation nor CK 20 expression could be observed. The DNA/RNA was re-extracted again from the same samples and later from paraffin embedded halves of the lymph nodes obtained from the pathologist. PCR was repeated twice for each sample; however, the result remained negative. Moreover, in one pN0-patient with K-ras negative primary tumor the K-ras marker of MDM was positive in para-aortal lymph node station (LN16). Generally it is expected that molecular abnormalities in primary site and metastatic lesions should be the same [43]. The given contradiction can be explained by tumor heterogeneity [65, 66].When K-ras mutation is not an early event in the tumor progression, both K-ras negative and K-ras positive cells subpopulations may coexist and successfully metastasize from the same tumor.

Genomic instability is often associated with tumor progression to metastatic competence and development of diversification continues after metastases have formed [67]. As a result some subpopulations of tumor cells may loose their primary genetic features during this process.

From the 28 patients who were involved in this study, one case presented pancreatic tail adenocarcinaoma and diffuse liver metastases at the time of surgery. This patient was excluded as not corresponding to R0 - criterion. The investigation of the patterns of lymphatic dissemination of the cancer of the pancreas head was carried out on 27 patients.

Nine patients (33%) represented distant lymph node metastases. Eight of these had local lymph node (anterior and/or posterior pancreato-duodenal) involvement. And one patient, who had distant lymph node metastasis had neither histological nor molecular signs of local lymph nodes involvement. This patient had one isolated distant lymph node metastasis along the celiac trunk (LN9). The lymph node was negative by molecular testing, despite the primary tumor was K-ras and CK 20 positive. This patient (N 25) also presented a distant nodal MDM in LN 14 (along the superior mesenteric artery). The patient died in the 6th month postoperatively. He developed a local recurrence in the pancreatic bed and peritoneum in spite of intensive adjuvant chemotherapy (Gemzar/Ukrain protocol) and immunotherapy with autologous dendritic cells. This case indicates that an atypical metastasation pattern, associated with genomic instability of the tumor, correlates with a dismal clinical prognosis.

Further molecular investigation revealed 5 of 27 patients (19%) who had signs of isolated distant nodal MDM. Obviously, circulating tumor cell in pancreatic cancer is not necessarily arrested in the primary lymph node since they can also pass through them without forming metastases. This corroborates the data obtained in the experimental model of Fisher and Fischer [68]. The “skipping” of the local lymph nodes in metastatic dissemination may also be explained by the presence of direct lymphatic channels between the pancreas and distant lymph nodes [69]. The present study also revealed that of 27 patients, 9 (33%) had pathohistologically proven distant nodal involvement, 20 patients (74%) had distant nodal MDM and the combination of all methods showed distant nodal involvement in 21 patients (78 %). Both molecular markers were concordant in the same tissue sample only in 5 pN0 patients and in 4 of them in para-aortal lymph nodes. The clinical relevance of this data will be discussed below.

The pattern of metastases and MDM spread among regional lymphatic system was not always the same for a given lymph node station. Here the MDM was considered if at least one molecular marker was positive. The most frequent site of distant nodal MDM was a group of para-aortal lymph nodes (LN 16). According to results of the molecular investigation of 18 patients with no evidence of distant lymph node metastases, the frequency of LN 16 MDM was 39% (7 patients). Of these, 6 patients had MDM only at this station. In 5 patients (19%) the lymph nodes along hepaticoduodenal ligament (LN12), and in 2 patients (7%) - lymph nodes along superior mesenteric artery were involved in MDM . In the report of Ando, who also used RFLP PCR for detection of K-ras codon 12 mutations, the frequency of LN 16 MDM for patients with pancreatic head carcinoma was 62% (8 from 13 patients) [22].

Of 12 patients with histologically tumor free distant lymph nodes in which MDM occurred, 5 patients (42%) were pN0 and 7 patients (58%) were pN1 according to JPS; 6 patients (50%) had tumor grade I-II and 6 (50%) – had tumor grade higher than II; 3 patients (33%) had lymphatic vessel invasion at the site of a primary tumor as opposed to 9 (67%), who had none; pT status according to UICC was I-II in 4 patients (25%) and II-IV – in 8 patients (75%). The Chi-square test was used to check the significance of these putative factors inducing MDM. The phenomenon of distant nodal MDM in patients with cancer of the pancreas head did not correlate with pathohistological pancreato-duodenal (local) lymph nodes status. Moreover, there was no statistical connection between the MDM in distant lymph nodes and lymphatic vessels invasion at the site of the primary tumor. Nor was there a

statistical connection found in relation to the histological grade and size or anatomical extent (pT) of the tumor.

Except for the detection of the MDM in distant lymph nodes and elucidating the factors of influence and the pattern of its spread, this study was focused on the possible correlation with clinical prognosis in patients with cancer of the pancreatic head. Of 27 R0-resected patients, 9 had distant lymph node metastases. The presence of metastases in distant lymph nodes tended to have a prognostic value for the decreased survival in this study ($p=0.0545$). Therefore these 9 cases were also excluded from further investigation of distant nodal MDM influence on clinical prognosis. In a comparison of survival rates between pN0-patients and patients with only local nodal metastases (data not shown) no significant difference was seen. These results correspond with the data produced by Ishikawa [4].

Consequently, among the 18 patients included in the investigation for clinical relevance there were pN0 patients, as well as patients with only local lymph node metastases. CK 20 marker alone and in combination with K-ras, when applied to all 18 patients, failed to show any correlation.

Furthermore, the presence of K-ras mutation in the primary tumor was a negative factor of influence on OS, which correlates with data from pancreas cancer patients published in recent reports [25, 70]. Out of 27 initial patients in the 22 K-ras positive patients, the actual 2-year survival rate was 32% (7patients) and the median survival – 17 (range, 1-40) months. Whereas, all 5 K-ras negative patients were alive at the time of the latest check, the actual 2-year survival for them was 60% (3 patients) while the median survival – was 34 (range, 4-40) months.

In 18 selected patients there were 14 K-ras positive and further analysis was carried out only on them. K-ras marker separately and in concordance with CK 20 in the same lymph node, also failed to reveal any considerable connection with clinical outcomes. These results are in apposition to the report of Tamagawa [26]. In his retrospective study 12 patients with pancreas head carcinoma, with only pT1-T3 tumor status (UICC), were included. All other criterion of inclusion were the same as in the current study. Like in our study, as a molecular marker for distant nodal MDM the mutation in K-ras codon 12 was detected by the enriched (RFLP – restriction fragment length polymorphism) PCR method. The 5-year survival of 5 patients without distant nodal MDM was 60% in contrast to 0 in 5 patients with MDM . The statistical analysis showed the differences to be significant.

Yamada in his study of 18 R0-patients with cancer of the pancreatic head [27] also reported significant difference in OS between 13 patients with and 5 patients without distant nodal MDM. Unlike our study, in Yamada's prospective study K-ras mutation in regional lymph nodes was detected using MASA (mutant allele specific amplification) method, which is also based on PCR.

Hosch et al. using Ber-EP4 antibody for immunohistochemical assay [24] presented data from 13 MDM positive and 5 MDM negative patients with pancreatic head cancer. The median PFS was, respectively, 7 versus >20 months and median survival 13 versus >20 months. Both differences were statistically significant.

Unlike others, in this study it was assumed that concordance of CK 20 expression with the presence of K-ras mutation in the same tissue sample (lymph node) would be an evidence for the presence of viable cancer cells. The K-ras mutation in codon 12 is pathognomonical for malignant genotype. Messenger-RNA of CK20, the expression of which is also characteristic for cancer phenotype, belongs to the class of unstable molecules that degrade rapidly in a non-viable cell. To prove this assumption the groups of patients were rearranged as described below.

Among patients with MDM who were diagnosed on the basis of a concurrence of both positive K-ras and CK20 markers in the same lymph node (concordant markers), there were 4 patients with MDM in LN 16. In this group there were no 2-year survivors observed

and the median survival and median PFS was 13 (range, 1-18) months. This group of patients was compared to the group of 4 patients with no signs of MDM . The 2-year actual survival and the 2-year PFS in the latter group reached 50% while the median survival and median PFS were 32 (range, 7-39) and 26 (range, 7-32) months, respectively. This difference in OS rate was considerable ($p=0.03$). When the patients of the LN16 MDM positive group were compared to the ten LN16 MDM negative patients (all remaining K-ras positive patients without concordant markers in LN16), the difference in the OS also was considerable ($p<0.005$). The 2-year actual survival and 2-year PFS in the latter group respectively was 50% and 60% while median survival and median PFS, respectively, were 32 (range, 1-40) and 26 (range, 1-37) months. Interestingly, the survival rates of patients with no signs of MDM and those without positive concordant markers in LN16 (being positive by only one of the markers) were almost identical. This fact underlines the practical value of the applied method and the importance of the LN 16 involvement for clinical prognosis for patients with pancreatic head cancer.

These results correspond to data from the report of Niedergethmann et al. [25] who used PCR method for detection of K-ras mutation in patients with cancer of the pancreatic head and compared the survival rates of 12 patients with LN 16 MDM to the survival of 30 patients who had no LN16 involvement. The survival of the former group was significantly poorer. Moreover, para-aortal lymph nodes diagnosed for K-ras mutation were independent prognostic markers in multivariate analysis of his data.

Nevertheless, in our study, despite the correlation between MDM in LN 16 and decrease in OS rate as revealed, we failed to establish such a correlation using only one molecular method as in the above mentioned reports [24-27]. This fact can be explained as follows: Taq-polymerase, the enzyme used for amplification of the DNA in PCR (which was also used in the present study), may introduce spontaneous replication errors in a range of 1 in 10 000 [71]. Cytokeratines sometimes may be expressed in reticulum cells of hyperplastic lymph nodes [72, 73], which leads to false positive results. Moreover, using RFLP/PCR or any other method of amplification of genomic cancer DNA , we can only detect the presence of it in the lymph node. However, this does not mean that the tumor cell is there. Cell free DNA is reported to circulate in peripheral blood of the cancer patients and these DNA fragments are 100 - 500 bp in size [74]. Consequently, these fragments may also appear in lymph nodes. Therefore, they can be detected by our RLFP PCR method because the distance between the primers in the first PCR step is 157 bps. Furthermore, Yamaguchi [43] emphasizes that tumor DNA may migrate from the original site to the lymph nodes and persist there up to 4 days. It is also reasonable to assume that phagocytes transport non-viable tumor cells to the lymph node.

The fact that LN16 was the most frequent site for both MDM and metastases dissemination can be a consequence of the fact that this lymph node station is the final site of lymphatic outflow from the abdomen [75]. The lymphatic flow from the pancreas head is directed to LN16 through a number of intermediate stations (LN14. LN12. LN9 etc. with the primary station being anterior and posterior pancreato-duodenal lymph nodes). LN16 is the origin of the thoracic lymphatic duct [76], which flows directly into venous blood circulation. This fact may be the reason that certain morpho-physiological peculiarities exist in the lymph nodes of this station, which contributes to a more pronounced antigen arrest and/or invasion of circulating cancer cells/clusters in subcapsular sinus of the lymph nodes of this station. Although the underlying mechanism remains virtually unexplored, it is known that tumor cell adhesion to various host cells such as lymphocytes, endothelial cells and platelets regulates invasion and proliferation [77-79]. The role of interaction of cancer cells integrins with lymph node stromal elements (vitronectin, fibronectin) also cannot be underestimated [80, 81].

However the presence of MDM , as well as metastases in the LN 16 makes the value of extended radical surgical resection questionable. Unfortunately, the molecular methods of

detection of MDM used in this study are too time-consuming for intraoperative diagnostics. Hence, this diagnostic procedure can be applied only postoperatively in order to identify and treat those patients who have a substantial chance for long term survival after curative resection.

The results of the present study, emphasize that the MDM in para-aortal lymph nodes station in patients with pancreatic head adenocarcinoma, when estimated by concordant K-ras/CK 20 markers, has a prognostic value in terms of OS. Patients with MDM in LN16 have considerably lower OS then those without MDM. Moreover, alike metastases in distant lymph nodes, MDM in LN16 tends to correlate with decreased PFS. The group of 9 patients with metastases in distant lymph nodes ($pN2^+$) showed considerably lower PFS rates and tended for lower OS ($p<0,06$), when compared to the 18 patients without metastases in distant lymph nodes ($pN2^-$). For $pN2^-$ and $pN2^+$ patients, respectively, the actual 2-year survival was 50% (9 patients) and 11% (1 patient); the 2-year PFS rate was 39% (7 patients) and 11% (1 patient), the median survival – 24 (range, 1-40) months and 7 (range,1-35) months, the median progression free 20 (range, 7-40) months and 6 (range, 5-35) months.

Other factors influencing the survival rates were adjuvant chemotherapy protocols. Patients treated according to Gemzar/ Ukrain [82, 83], but not ESPAC 1 [84, 85] protocol had essentially higher PFS than patients not treated with chemotherapy. The median PFS for these groups of patients were, respectively, 20 (range, 5-40) months, 13 (range, 8-28) months and 7 (range, 4-18) months. Furthermore, a group of 15 patients treated with Gemzar/ Ukrain protocol had considerably higher OS than in a group of 8 patients not treated with chemotherapy ($p=0.01$). The 2-year actual and median survival were, respectively, 60% and 32 (range, 2-40) months for Gemzar/ Ukrain group, and 0 and 10 (range, 1-19) months for patients not treated with chemotherapy.

The fact that more than 50% of patients in this study were treated according to Ukrain/Gemzar protocol, which considerably increases the PFS, could be the reason we were unable to establish any correlation between MDM appearance and recurrence rate.

5. SUMMARY

Despite the considerable progress in our understanding of the pathogenesis and the novel approaches in treatment of pancreatic cancer, the outcome of the disease is still dismal**.** More than 40% of the patients develop local regional recurrence within 6 months and 60-80% do so within 12 months of surgical treatment. The recurrences are seen in some pathohistologically node-negative cases shortly after curative resection. Early lymphatic spread is assumed to be one of the main reasons for this. Obviously routine histological examination with hematoxylin-eosin staining fails to detect cancer involvement in some lymph nodes.

The **purpose** of this work was:

1. To test the efficacy of two molecular methods: Restriction Fragment Length Polymorphism Polymerase Chain Reaction (RFLP PCR) for detection of mutation in codon 12 of oncosupressor K-ras gene and Reverse Transcription (RT) PCR for CK 20 expression as markers for molecular detection of occult metastases (MDM) in patients with adenocarcinoma of the pancreas. *This combined method should reflect not only the appearance of tumor genetic material in the lymph node, but also its viability, due to detection of CK20 messenger RNA, which is a short-living molecule.*
2. To analyze the correlation between the nodal MDM appearance and different morphological parameters of the primary tumor and lymph node status in patients after curative resection (R0 patients) with adenocarcinoma of the head of the pancreas.
3. To investigate the pattern of MDM in different distant lymph nodes stations in R0 patients with adenocarcinoma of the head of the pancreas.
4. To analyze the influence of distant nodal MDM and another putative factors on progression free and overall survival rate in R0 patients with adenocarcinoma of the head of the pancreas.

The MDM phenomenon in the regional lymph nodes was studied by means of routine histopathological examination and two molecular methods:

1. RT PCR for detection of CK 20 mRNA expression
2. RFLP PCR for detection of K-ras codon 12 point mutation.

Methods: The study was carried out on 28 patients. Altogether there were 531 lymph nodes histologically investigated in this study. Of these 226 (42%) were regional lymph nodes submitted only to histological investigation. The rest of 305 lymph nodes were hemisected and tested histologically, as well as for the presence of K-ras mutation and 239 of the latter were tested for CK20 expression. Histological examination showed tumor invasion in 12.9% regional and in 11.0% lymph nodes. Application of molecular methods (K-ras and CK20 together) increased these indices up to 25.9% for the distant lymph nodes in all patients.

Results:

1. The sensitivity of K-ras marker in this study was 89.7% . The application of CK 20 allowed increasing the sensitivity up to 96.7 %, although the application of CK 20 marker alone presented very high level of false positive results 61.5%.
2. The distant nodal MDM in patients with pancreatic cancer is a phenomenon independent from histological tumor grade, lymphovascular invasion at the site of a primary tumor, pT and pN status.
3. The pattern of MDM and metastasis spread around the regional lymphatic system are not always the same. In spite of that, the most frequent site of MDM and metastasis spread around regional lymphatic system was the para-aortal lymph nodal station and the lymph nodes along the a. mesenterica sup. In one patient with tumor-free local lymph nodes one metastasis was detected in the lymph node along the celiac trunk.

4. Among patients with MDM who were diagnosed on the basis of a concurrence of both positive K-ras and CK20 markers in the same lymph node (concordant markers), there were 4 patients with MDM in para-oartic lymph nodes (LN16). In this group there were no 2-year survivors observed and the median survival and median PFS were both equal to 13 (range, 1-18) months. This group of patients was compared to the group of 4 patients with no signs of MDM . The 2-year actual survival and the 2-year PFS in the latter group reached 50% while the median survival and median PFS were 32 (range, 7-39) and 26 (range, 7-32) months, respectively. This difference in OS rate was considerable. When the patients of the LN16 MDM positive group were compared to the ten LN16 MDM negative patients (all remaining K-ras positive patients without concordant markers in LN16), the difference in the OS was considerable. The 2-year actual survival and 2-year PFS in the latter group respectively was 50% and 60% while median survival and median PFS, respectively, were 32 (range, 1-40) and 26 (range, 1-37) months.

Other factors decreasing the survival chances of patients with pancreatic head adenocarcinoma include the presence of metastases in distant lymph nodes, the presence of K-ras mutation in the primary tumor and the absence of adjuvant CT treatment.

Conclusion:

This study revealed that CK 20 marker should be applied only as supplemental in combination with K-ras marker for the detection of distant nodal MDM in patients with pancreas cancer.

The most frequent site of metastasation among distant lymph nodes were Ln16 and LN 14 .The phenomenon of “skipping” the local lymph nodes is described.

The MDM appearance is independent from clinico-morphological parameters of the primary tumor.

The PCR method of concordant K-ras and CK 20 markers in paraaortic lymph nodes was demonstrated to be effective in clinical prognosis evaluation of R0- resected patients with the cancer of the pancreatic head. The presence of MDM in para-aortal lymph nodes is identical to metastatic lesion of that station in terms of clinical prognosis of the patients

The protocol of adjuvant chemotherapy considerably influences the overall and progression free survival of the patients with pancreatic cancer.

The data concerning clinical relevance of MDM was obtained from 14 patients. The question of MDM influence on clinical prognosis requires further investigation in larger population of patients.

APPENDIX 1. UICC TNM Classification of Malignant Tumors. Fifth Edition. 1997.

Table 20. UICC staging criteria.

	T	N	M
Stage I	T1, T2	N0	M0
Stage II	T3	N0	M0
Stage III	T1, T2, T3	N1	M0
Stage IV A	T4	Any N	M0
Stage IV B	Any T	Any N	M1

T – Primary Tumor
T1- tumor limited to the pancreas, 2cm or less in greatest dimension
T2 – Tumor limited to pancreas, more then 2 cm in greatest dimension
T3 – Tumor extends directly into any of the following: duodenum, bile duct, peripancreatic tissues[1]
T4 – Tumor extends directly into any of the following: stomach, spleen, colon, adjacent large vessel[2]

Note: 1. Peripanreatic tissues include the surrounding retroperitoneal fat (retroperitoneal soft tissue or retroperitoneal space), including mesentery (mesenteric fat), mesocolon, greater and lesser omentum, and peritoneum. Direct invasion to bile ducts and duodenum includes involvement of ampulla of Vater.
2. Adjacent large vessels as the portal vein, coeliac artery, and superior mesenteric and common hepatic arteries and veins (not splenic vessels).

N – Regional Lymph Nodes
N0 – no regional lymph nodes metastases
N1a – metastasis in a single regional lymph node
N1b – metastasis in multiple regional lymph nodes

M- Distant Metastasis
M0 – no distant metastasis
M1 – distant metastasis

Table 21. Residual Tumor (R) Classification

RX	Presence of residual tumor can not be assessed
R0	No residual tumor
R1	Microscopic residual tumor
R2	Macroscopic residual tumor

APPENDIX 2. JPS Classification of Regional Lymph Nodes in Pancreatic Cancer.

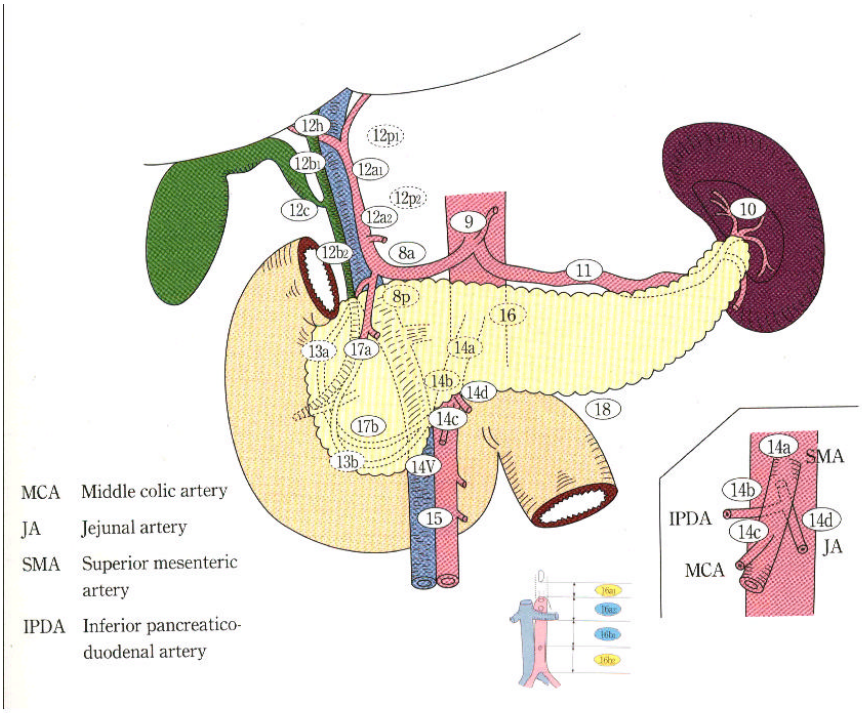

Fig.1. Regional lymph nodes in pancreatic cancer

LN 8 – around the *arteria hepatica communis* (a- anterior, b-posterior)
LN 9 – around the *truncus coeliacus*
LN10 - at the hilus of the spleen
LN11 - along the *arteria lienalis*
LN12 – at *ligamentum hepatoduodenalis* (a- along *arteria hepatica propria*; b- along the common bile duct; c- near the cystic duct; h - at the hilus of the liver; p – along portal vein)
LN13 – posterior pancreato-duodenal lymph nodes (a- superior, b- inferior)
LN14 – along the *arteria mesenterica superior* (a- root of mesenteric artery; b- root of inferior pancreatoduodenal artery; c- root of middle colic artery; d- root of jejunal artery)
LN15 – along the *arteria colica media*
LN16 - paraaortal lymph nodes
LN17 – anterior pancreatiduodenal lymph nodes (a- superior, b-inferior)
LN18 – lymph nodes at the lower edge of the tail of the pancreas

LN8, LN12 (distal), Ln13 and LN17 are considered as local lymph node stations in lymphodissemination of the cancer of the pancreatic head and correspond to N1 in JPS classification, the remaining regional lymph node stations correspond to N2.

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