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Proteins of the retinoblastoma pathway, FEN1 and MGMT are novel potential prognostic biomarkers in pancreatic adenocarcinoma

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Abstract

Background

We studied the expression of some major proteins involved in cell-cycle regulation and DNA repair, the roles of which are not well known in pancreatic ductal adenocarcinoma (PDAC), but which have a significant impact on carcinogenesis of many other cancers.

Methods

We immunohistochemically assessed expression levels of the cell-cycle regulators Rb1, p16 and cyclin-dependent kinase 4 (CDK4), and the DNA repair enzymes O6-methylguanine-DNA-alkyltransferase (MGMT) and flap endonuclease-1 (FEN1) separately in malignant tissue and benign tissue from resection margins in 102 cases of PDAC. Nearly all (95.1%) patients had undergone pancreaticoduodenectomy.

Results

The studied proteins showed wide but somewhat variable expression in both benign and malignant pancreatic tissues. Strong CDK4 expression in islets of Langerhans predicted poor relapse-free survival (RFS) (HR 2.874; 95% CI 1.261–6.550; p=0.012) and within T3–4 tumors CDK4 expression in adenocarcinoma cells also predicted poor disease-free survival (DFS) (RR 2.148; 95% CI 1.081–4.272; p=0.029). Strong MGMT expression was associated in N1 patients with weak local relapse-free survival (RFS), DFS and overall survival; all significantly in Cox regression analysis. FEN1 was also an independent predictor of decreased DFS (in the whole study population) and worse RFS (in the patients with T3–4 tumors).

Conclusions

Major cell-cycle regulators and DNA repair enzymes display notable prognostic roles in PDAC, especially in the most aggressive cases. Based on levels in other tumor types, their expression may also have predictive significance, but further studies are required to evaluate this.

Abbreviations

Rb = Retinoblastoma-associated protein-1

CDK4/6 = Cyclin-dependent kinases 4 and 6

MGMT = O6-methylguanine-DNA-alkyltransferase

FEN1 = Flap endonuclease-1

PDAC = Pancreatic ductal adenocarcinoma

DFS = Disease-free survival

RFS = Relapse-free survival

OS = Overall survival

TLS = Tertiary lymphoid structures

Keywords: cell cycle; DNA repair; immunohistochemistry; pancreatic cancer; survival

Introduction

The retinoblastoma (Rb) pathway is one of the key elements of cell-cycle regulation (1). During G1, cells react to incoming extracellular signals by advancing to cell division or receding to a resting state (G0) (2). Mutations and overexpression of the *RB* gene are linked to various cancers such as non-small cell lung cancer and breast cancer (3).

Cyclin-dependent kinases 4 and 6 (CDK4/6) are needed in the phosphorylation of Rb1 protein, leading to its inactivation, release of E2F transcription factors and consequently the expression of genes required for progression of the cell cycle and entry to the S phase (4). Elevated CDK4/6 activity promotes tumor growth (5). The protein p16ink4 acts as a tumor suppressor by binding to

CDK4/6 and it prevents the catalytic activity of cyclin D1-CDK4/6 holoenzymes (6). Targeting CDK4/6 in combination with the use of antiestrogens or aromatase inhibitors is a new method in the treatment of advanced estrogen receptor-positive breast cancer and clinical studies on CDK4/6 inhibitors in connection with many cancer types are ongoing (7) (8) .

DNA replication and repair are crucial for maintaining genome stability. The DNA repair enzyme O6-methylguanine-DNA-alkyltransferase (MGMT) protects the genome by removing mutagenic alkyl groups from the O6 position of guanine, thus protecting cells from exogenous carcinogens. If the alkyl group is not removed, O6 guanine is read erroneously as adenine (A) and it pairs with thymine (T) in DNA replication. Therefore, it is possible that unrepaired lesions may cause mutation in proto-oncogenes. Inactivation of MGMT, usually by methylation of the gene-regulatory region, can thus trigger cell transformation into cancer cells (9). Different tumors have been noted to be heterogeneous in MGMT expression (10). The results of several studies suggest that MGMT has a key role in resistance to alkylating chemotherapy (11).

Flap endonuclease-1 (FEN1), a 43-kDa protein, is a structure-specific and multifunctional nuclease (12). It is critical during DNA long-patch base excision repair (LP-BER) and Okazaki fragment maturation during replication. FEN1 also plays essential roles in rescue of stalled replication forks, maintenance of telomere stability, and apoptosis (13) (14). Dysregulation of FEN1 can result in damaged genetic information coded in DNA and disarray in programmed cell cycles (15). Increasing evidence shows that FEN1 plays a pivotal role in carcinogenesis and FEN1 overexpression has been detected in several malignancies such as testis-, non-small cell lung- and brain cancers (16) (17).

We immunohistochemically assessed expression of the cell-cycle regulators CDK4, p16 and Rb1, and the DNA repair enzymes MGMT and FEN1 in PDAC tissue and separately in benign tissue

from surgical resection margins. Our primary aim was to evaluate the possible prognostic value of these poorly studied proteins and associations with traditional prognostic factors in human PDAC.

Materials and methods

Patients and samples

The material consisted of 102 surgical PDAC samples before the initiation of any treatment. All patients were diagnosed and treated at Oulu University Hospital in 1993–2015 and the cohort consisted of samples available from this time period. Owing to the lack of reliably representative material, FEN1 was assessed in only 81 cases. Most (97; 95.1%) of the patients underwent pancreaticoduodenectomy (Table 1). Immunostaining results were assessed both in adenocarcinoma cells and separately in benign pancreatic tissues from resection margins, when available (n=21 to 86 depending on staining). In addition, we took care to examine peritumoral tissue to detect specific peritumoral immunostaining. The specimens had been fixed in neutral formalin, embedded in

paraffin blocks and stored at the Department of Pathology, Oulu University Hospital. Fifty (49.0%) of the patients had been diagnosed in or after 2010. During the follow-up period (median 15 months) 72 patients (70.6%) died of pancreatic cancer. Diagnoses were reviewed by a specialist pathologist and evaluation of immunostaining was performed by experienced histopathologist (KMH) and JI). Exact and updated patient data was acquired from medical records. During the evaluation of immunostaining, the investigators were blind to the clinical patient data. Pathology TNM staging data was available in 99 (97.1%) cases and clinical TNM staging alone in two (2.0%) cases. In one case, reliable TNM staging was absent.

Immunohistochemistry

The PDAC samples and benign pancreatic tissue from resection margins were fixed in formalin and embedded in paraffin. Sections of 3.5 μm thickness were rehydrated in a descending series of ethanol solutions and deparaffinized in xylene. In staining for Rb1, FEN1 and MGMT, antigen retrieval was carried out in a microwave oven in citrate buffer at pH 6 for 17 minutes for Rb1 and 12 minutes for FEN1 and MGMT. In staining for CDK4 and p16 the samples were also pretreated in a microwave oven, but in citrate buffer at pH 9 for 17 minutes. After that, the samples were cooled at room temperature for 20 minutes. Next, in all cases, endogenous peroxidase activity was blocked with Dako REAL™ Peroxidase-Blocking solution (Dako S2023, Dako Denmark A/S, Glostrup, Denmark) for 15 minutes. The samples were incubated with primary antibodies (Table 2) at +4 ºC for 30 minutes for p16 and CDK4 staining, for 60 minutes for Rb1 and FEN1 staining, and overnight for MGMT immunostaining. Next, the slides were incubated with secondary biotinylated antibodies (Dako S2023, Dako Denmark A/S, Glostrup, Denmark) and immunostaining was carried out with a NovoLink Polymer Detection System (Leica Biosystems, Newcastle, UK) or a Dako REAL™ EnVision™ Detection System (Dako Denmark A/S, Glostrup, Denmark) according to the instructions of the manufacturers. Between stages of the immunostaining procedure, the slides were

washed with Tris-buffered saline (TBS). The chromogen used was 3,3'-diaminobenzidine and the slides were counterstained with Mayer's hematoxylin and finally mounted. Negative controls were carried out using same procedures omitting primary antibody.

Statistical analyses

For statistical analyses, immunostaining intensity (0–3) was multiplied by the percentage of stained cells out of all PDAC cells (0–100%), resulting in a continuous variable of 0–300. Both intensity and the extent of immunostaining were separately evaluated in nuclei and cytoplasm, and separately in adenocarcinoma cells and cells of exo- and endocrine pancreas from resection margins. The Mann–Whitney test was used to determine the significance of the results, with the exception of survival analyses, where the continuous variable was divided into two classes (low or high expression) based on the median expression of each variable.

Grade was divided into well-to-moderate differentiation or poor differentiation and T-class was handled in statistical analyses as T1–2 or T3–4. Associations between protein levels and patient survival were analyzed by using the Kaplan–Meier method with the log-rank test. Disease-free survival (DFS) was calculated from the date of diagnosis to the date of the first confirmed relapse, either local or distant. Relapse-free survival (RFS) was defined as the time from diagnosis to local relapse. Overall survival (OS) was calculated from the date of diagnosis to the time of death from any cause. Cox regression analysis was applied in multivariate analysis. Statistical analyses were carried out by using IBM SPSS Statistics 24.0.0.0 software (SPSS, Armonk, NY, USA) and the results were considered significant if the two-sided p-value was <0.05.

Results

Staining patterns in malignant tissue in PDACs

Expression of p16 was detected in less than half of the cases, both in nuclei and cytoplasm. When present, nuclear staining intensity ranged from weak (+) to strong (+++) and in most cases the extent of immunostaining was 5–50%. Cellular staining intensity varied from weak (+) to strong (+++), but only 4 samples showed strong immunopositivity. The extent of cytoplasmic immunostaining ranged from 5 to 100%. Positive staining was also detected in tumor-associated fibroblasts (n=21). In these samples 56.7% of the cases showed no immunostaining and 5 cases were not evaluable because of exhaustion of the blocks or the occurrence of non-representative areas.

CDK4 expression was mainly detected in nuclei, being identified in 55 (55.0%) of the cases. Nuclear intensity varied from weak (+) to strong (+++). Cytoplasmic CDK4 was seen in 17 of the cases and the intensity was mainly weak in the evaluable cases. The extent of nuclear CDK4 staining varied from 5% to 50%. The extent of cytoplasmic CDK4 staining ranged between1%–100%. A peritumoral stromal CDK4 immunoreaction was detected in 37% (n=37) of the cases. Most samples (n=75) showed weak (+) or moderate (++) nuclear Rb1 positivity and only 13 of the cases showed cytoplasmic staining. The magnitude of the immunoreaction varied between 1–100% both in nuclear and cytoplasmic staining. Most cases also showed tumor-associated Rb1 in the stroma (50.5%) and in lymphocytes (55.6%). Owing to exhaustion of the blocks or the occurrence of non-representative areas, CDK4 and Rb1 were not evaluable in 2 and 3 cases, respectively.

Weak (+) to strong (+++) nuclear MGMT staining was detected in 82 cases (82.0%) and weak (+) to moderate (++) staining in the cytoplasm in the majority of these cases. In nuclei, the magnitude of staining ranged from 5 to 100% but in cytoplasm the extent was 100% in every sample. Only two of the cases were not evaluable. All of the samples showed nuclear FEN1 staining and the intensity varied between weak (+) and strong (+++). About two thirds (67.6%) of the cases also showed cytoplasmic immunoreactions but the intensity was mainly weak. The extent of FEN1 staining ranged from 1 to 90% in nuclei but in cytoplasm it was 100%. In addition, 72 out of 74 cases (97.3%) showed FEN1 immunopositivity in tumor-associated lymphocytes, but interestingly 8 of the cases 10.8% also showed immunoreactivity in tertiary lymphoid structures (TLSs). Seven of the cases were not evaluable because of exhaustion of the blocks or the occurrence of non-representative areas.

Staining patterns in benign tissue from resection margins

Expression of p16 was detected both in nuclei and cytoplasm in benign pancreatic exocrine tissue in a minority of the cases (n=21, 33.3%) and the intensity varied mostly from weak (+) to moderate (++). Forty-two cases showed no immunostaining at all. The extent of nuclear and cytoplasmic staining ranged from 1 to 20%. In contrast, 58.7% (n=37) of the cases showed immunostaining in endocrine cells of islets of Langerhans. Thirty-nine cases were not evaluable.

Exocrine pancreatic tissue showed no CDK4 immunopositivity, but in islets of Langerhans, CDK4 immunoreactivity was detected in 40 cases (46.5%). Similarly, only one case (1.2%) showed (weak) Rb1 staining in exocrine pancreatic tissue, but in endocrine tissue in islets of Langerhans, Rb1 immunoreactivity was observed in the majority of cases (n=73, 86.9%). Weak (+) or moderate (++)

MGMT staining was observed in 27 (79.4%) cases in nuclei and in 23 of the cases in cytoplasm in exocrine pancreatic tissue. The extent of MGMT staining varied from 5 to 80% in nuclei, but in the cytoplasm, the magnitude was 100% in all cases. In exocrine pancreatic tissue, nuclear FEN1 expression was detected in all cases and 59% of the cases also showed cytoplasmic staining. Sixteen to 18 cases were not evaluable because of exhaustion of the blocks or the occurrence of non-representative areas.

Association with clinical parameters

Expression of Rb1in the nuclei of adenocarcinoma cells showed an inverse correlation with the number of metastatic lymph nodes (p=0.024; r = -0.279). High-level nuclear MGMT1 expression in benign pancreatic cells from resection margins was associated with lower T-class (p=0.048). Both strong nuclear and cytoplasmic p16 immunostaining in pancreatic cancer cells were associated with better differentiation (p=0.006 and p=0.005). High-level cytoplasmic CDK4 expression in pancreatic cancer cells was associated with nodal involvement (p=0.043).

Survival analysis

Expression of FEN1 at any level in TLSs in peritumoral tissue was associated with shorter DFS (p=0.007) and shorter RFS (p=0.035). In multivariate analysis (Table 4) FEN1 was the most significant predictor of DFS (RR 2.619; 95% CI 1.132–6.059; p=0.025) when metastatic lymph node involvement (RR 1.905; 95% CI 0.999–3.633; p=0.050) and T-class (RR 1.344; 95% CI 0.688–2.627; p=0.387) were also included in the model. In addition, in multivariate analysis FEN1 was also the most significant predictor of RFS (RR 3.758; 95% CI 1.148–12.299; p=0.029) when

metastatic lymph node involvement (RR 1.284; 95% CI 0.572–2.881; p=0.545) and T-class (RR 2.263; 95% CI 0.885–5.783; p=0.088) were also included in the model.

In benign pancreatic tissue from resection margins, CDK4 positivity in islets of Langerhans was associated significantly with shorter RFS (p=0.001). In multivariate analysis CDK4 positivity in islets of Langerhans was the most significant predictor of RFS (RR 2.874; 95% CI 1.261–6.550; p=0.012) when metastatic lymph node involvement (RR 1.285; 95% CI 0.584–2.827; p=0.584) and T-class (RR 1.289; 95% CI 0.547–3.041; p=0.562) were also included in the model. When considering only patients with T3–T4 tumors, high-level nuclear CDK4 expression in PDAC cells was associated with shorter DFS (p=0.049). In Cox regression analysis this was a more significant predictor of decreased DFS than nodal involvement (for CDK4, RR 2.148; 95% CI 1.081–4.272; p=0.029 and for N-class, RR 2.102; 95% CI 1.009–4.380; p=0.047). Likewise, although MGMT was not connected to any survival parameters in the whole population, when we studied patients with T3–T4 tumors, high-level nuclear MGMT expression in PDAC cells tissue was associated with shorter OS (p=0.042), and in multivariate analysis this was more significant than nodal status. Again, when considering only the patients with nodal involvement, high-level nuclear MGMT expression in PDAC cells was associated with significantly shorter OS (p=0.014) and RFS (p=0.017), and nearly significantly associated with shorter DFS (p=0.063). All these analyses were significant when they were included in the Cox regression model along with tumor size.

Expression of p16 in tumor-associated fibroblasts was associated with shorter RFS in univariate analysis (p=0.019), but the results of multivariate analysis did not support this finding.

Discussion

According to our results, proteins involved in cell-cycle regulation and DNA repair seem to have central roles in the aggressiveness of PDAC. Although relatively widely studied in other carcinomas, most of the proteins assessed in our study have been poorly studied in PDAC. One of the strengths of the current study was the careful evaluation of expression in benign pancreatic tissue, which, according to our results, should be a part of standard evaluation of these biomarkers. We also had homogeneously treated (mainly with curative intention) single-institution material, which increases the plausibility of the reported results. Weaknesses of our setting may include the relatively long time period between cases. Also, the number of cases could have been larger.

Increased CDK4/6 activity initiates cell division and tumor growth by preventing the function of the tumor suppressor protein Rb1. Our results from PDAC cases suggest that CDK4 overexpression in PDAC cells increases the possibility of nodal involvement, while strong CDK4 expression in the

islets of Langerhans was connected with poor local relapse outcome (RFS). In addition, in the most extensive tumors (T3–4), strong CDK4 expression in adenocarcinoma cells was associated with decreased DFS. Preliminary data from some ongoing clinical trials shows that CDK4/6 inhibitors also have activity in pancreatic cancer(8) (18). As far as we know, there are no previous studies on the prognostic value of CDK4 in PDAC, but our current results are in line with data concerning other cancer types (19). The association between expression of CDK4 in cells of islets of Langerhans and poor local relapse outcome seems perplexing on the face of it. This association probably is not causal but reflects the regeneration of islet cells after tissue destruction, an effect which may extend to ductal cancer cells (20). Although it has not been assessed in the context of PDAC, CDK4 is vital to regulate physiological pancreatic islet development (21).

Nuclear Rb1 expression in adenocarcinoma cells showed an inverse correlation with the number of metastatic lymph nodes, and both strong nuclear and cytoplasmic p16 immunostaining in pancreatic cancer cells was associated with better differentiation. This emphasizes the tumor suppressive value of these two proteins in PDAC.

The DNA repair enzyme MGMT is physiologically expressed in all human cells, but different tumors show heterogeneous MGMT expression (9). MGMT overexpression has been described in various malignancies such as colon cancer, gliomas, lung cancer, breast cancer, leukemia, lymphomas and myeloma (22). On the other hand, loss of MGMT expression through epigenetic MGMT gene silencing due to promoter methylation has been reported, especially in glioblastoma multiforme, where MGMT status is currently a significant predictive factor in clinical practice involving temozolomide treatment (23) (24). In a small (n=30) cohort of PDAC patients treated with FOLFIRINOX combination chemotherapy, MGMT expression had a tendency to reflect poorer progression-free survival and OS (25). In another study, a specific single nucleotide

polymorphism of MGMT (IVS-44836G>A) predicted dismal overall survival in PDAC patients (26).

Although MGMT was not of prognostic significance in the whole patient population in our material, in the most extensive tumors (T3–4) and in those with nodal involvement MGMT expression in cancer cells was a highly significant predictor of RFS. Among the patients with nodal involvement, elevated nuclear MGMT expression was also associated with DFS and OS. This may be explained by effective DNA repair where there is oxidative stress in cancerous cells, leading to avoidance of apoptosis. Another hypothesis is that there could be an "excess" effective function of MGMT which paradoxically also protects cancer cells, in relation to a particular adjuvant treatment. Due to our limited data on adjuvant treatment, we could not assess this aspect. Nevertheless, preclinical evidence suggests a role of MGMT in gemcitabine resistance, in a survivin-mediated manner (27).

One of the most intriguing results was the highly significant interaction between FEN1 expression in tertiary lymphoid structures and shortened DFS and RFS. Most of the samples did not have any FEN1 expression in TLSs, but when present, the prognostic power even exceeded that of TN classification. A previous study has suggested intratumoral TLS as a favorable prognostic indicator in PDAC (28). Although we did not compare the presence of TLS with survival, these results underline the importance of microenvironment and local immune response in PDAC. They also may emphasize the immunogenic phenotype of PDAC (29). In our material, TLS were nearly always intratumorally located. FEN1 associates with poor outcomes also in ovarian and breast cancers (13). At least *in vitro*, FEN1 also confers chemoresistance against cisplatin which can be

overcome with FEN1 inhibitor (17). Although we did not have adjuvant chemotherapy data, it would be interesting to assess if FEN1 is also linked to PDAC chemotherapeutic agents.

Conclusions

As discussed above, the expression levels of MGMT in the most high-risk cases and FEN1 in the whole study population emerged as potential prognostic indicators of worse outcome in PDAC after surgical treatment. We also linked CDK4 expression with worse prognosis, while Rb1 and p16 seem to have only minor roles in PDAC. There were surprisingly few associations between the studied proteins and traditional clinicopathological parameters, which suggests that the reported associations with survival are independent of tumor size and nodal involvement. Further studies are required not only to assess these issues and confirm the current results, but also to evaluate if FEN1 and MGMT could serve as predictive factors for gemcitabine adjuvant therapy.

Ethical declaration

This study was approved by the Local Ethics Committee of the Ostrobothnia Hospital District (114/2011) and the National Supervisory Authority for Welfare and Health (Dnro 9580/05.01.00.06/2010)

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1. Kaplan–Meier curves showing disease-free survival (DFS) and relapse-free survival (RFS) according to FEN1 expression in tertiary lymphoid structures (TLSs) in peritumoral tissue (A, B). Kaplan–Meier curves showing RFS according to CDK4 expression in islets of Langerhans (C) and DFS according to CDK4 expression in cancer cell nuclei (D). Kaplan–Meier curves showing overall survival (OS) in T3–T4 cases according to MGMT expression in cancer cell nuclei (E). Kaplan–Meier curves showing OS, DFS and RFS in node-positive cases according to MGMT expression in cancer cell nuclei (F, G, H). Kaplan–Meier curves showing RFS according to p16 expression in tumor-associated fibroblasts (I).

Figure 2. Any (A) and no (B) FEN1 expression in tertiary lymphoid structures (TLSs). Any (C) and no (D) CDK4 expression in islets of Langerhans in the resection margin. High (E) and low (F) nuclear CDK4 expression in PDAC cells. High (G) and low (H) nuclear MGMT expression in PDAC cells. Any (I) and no (J) p16 expression in tumor-associated fibroblasts. (Low magnification ×10)

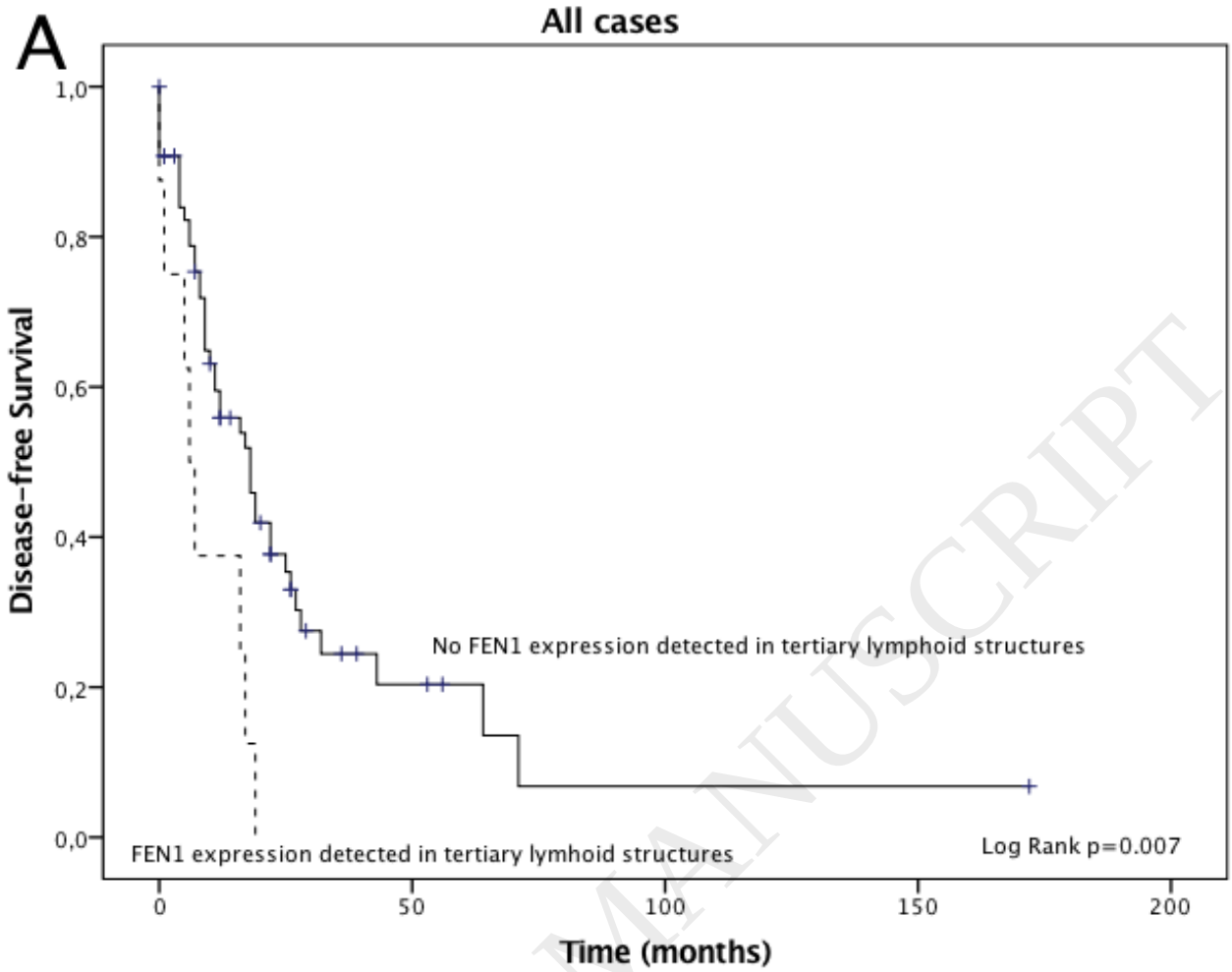


Fig 1A

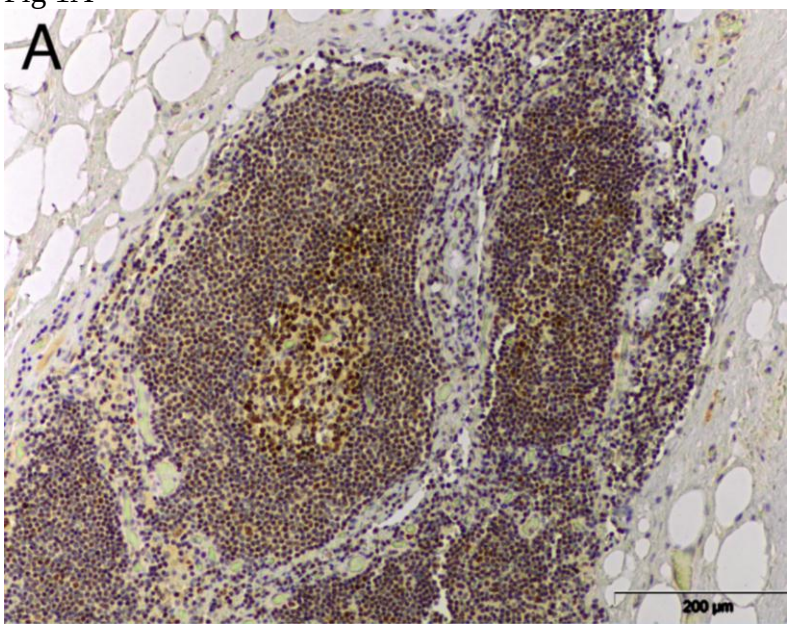


Fig B

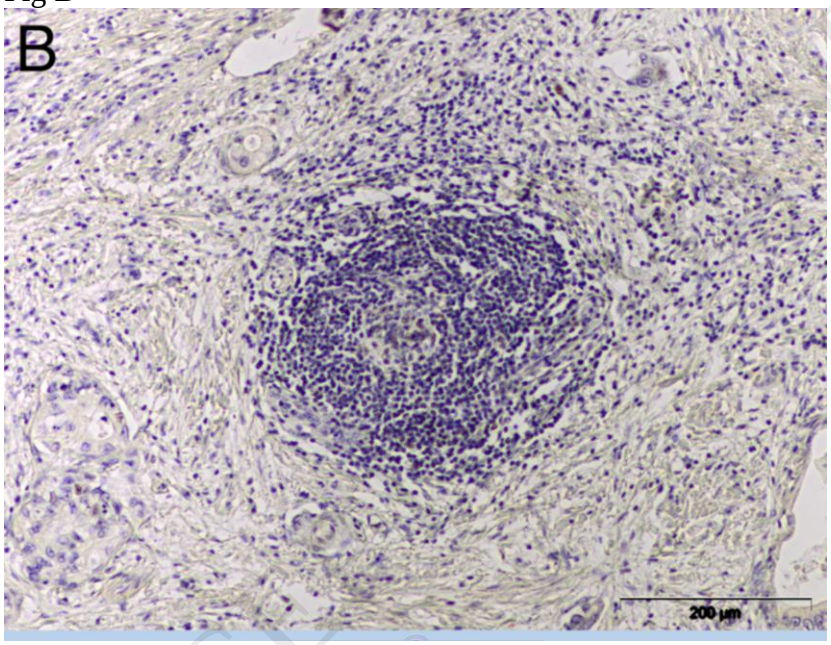
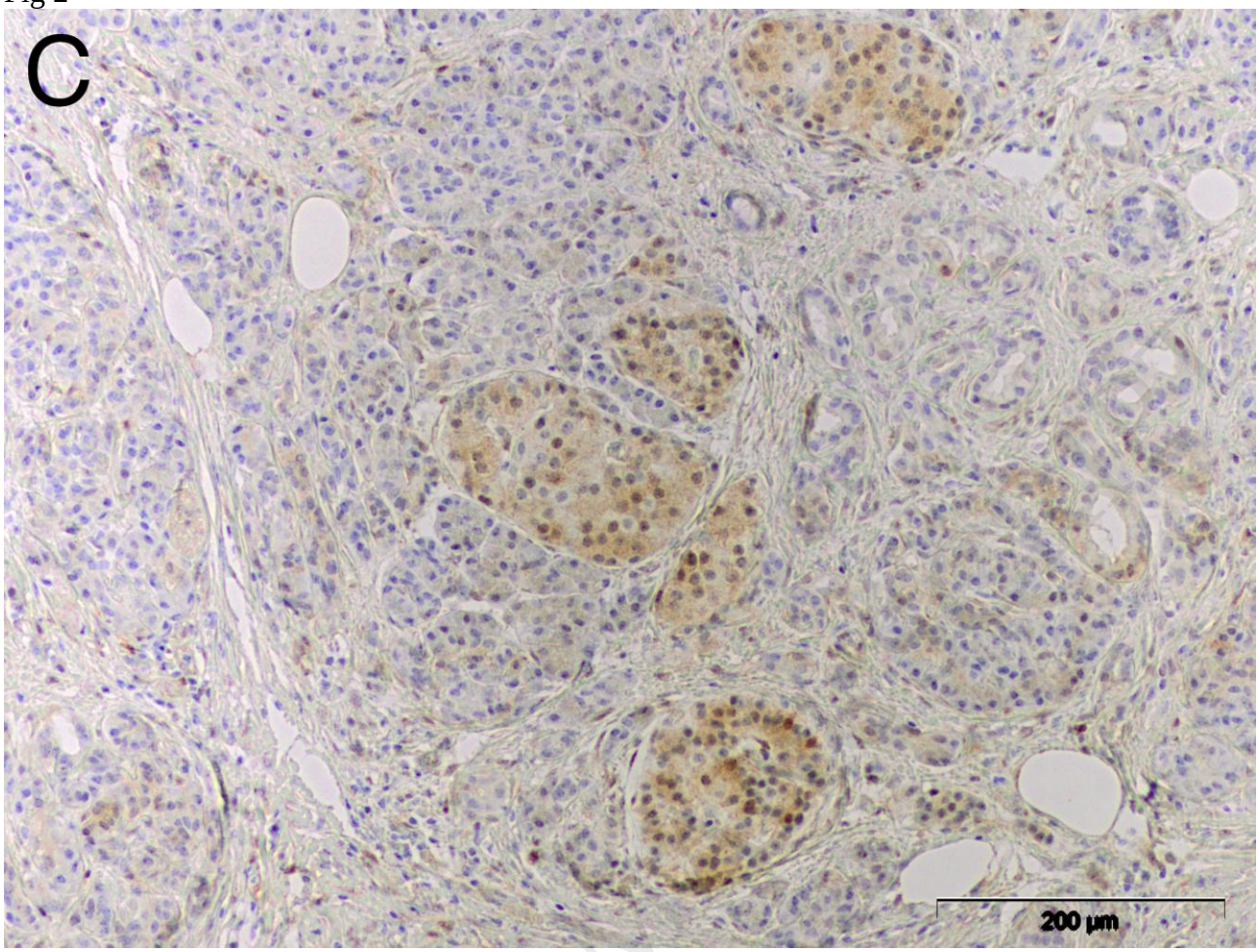
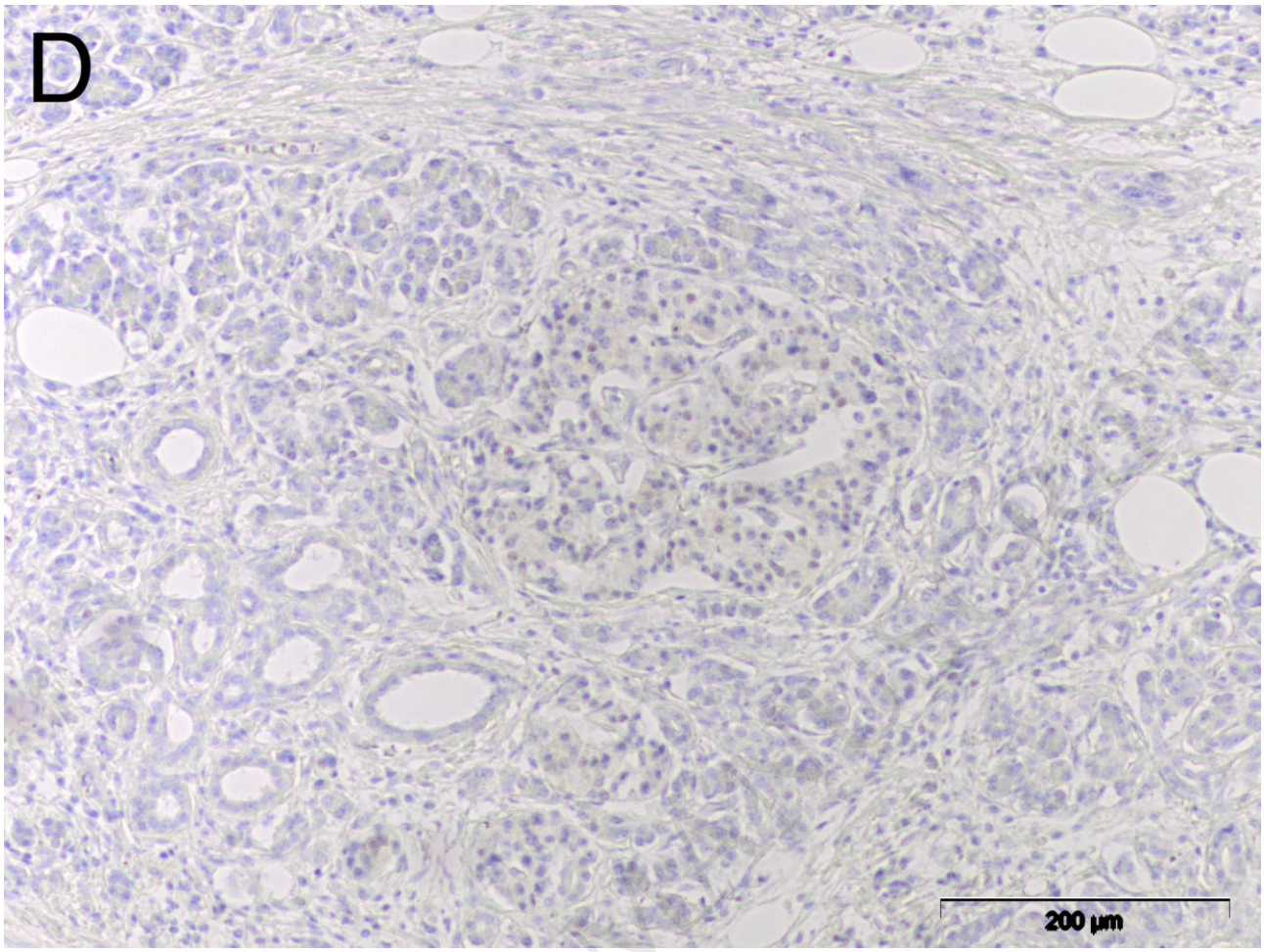
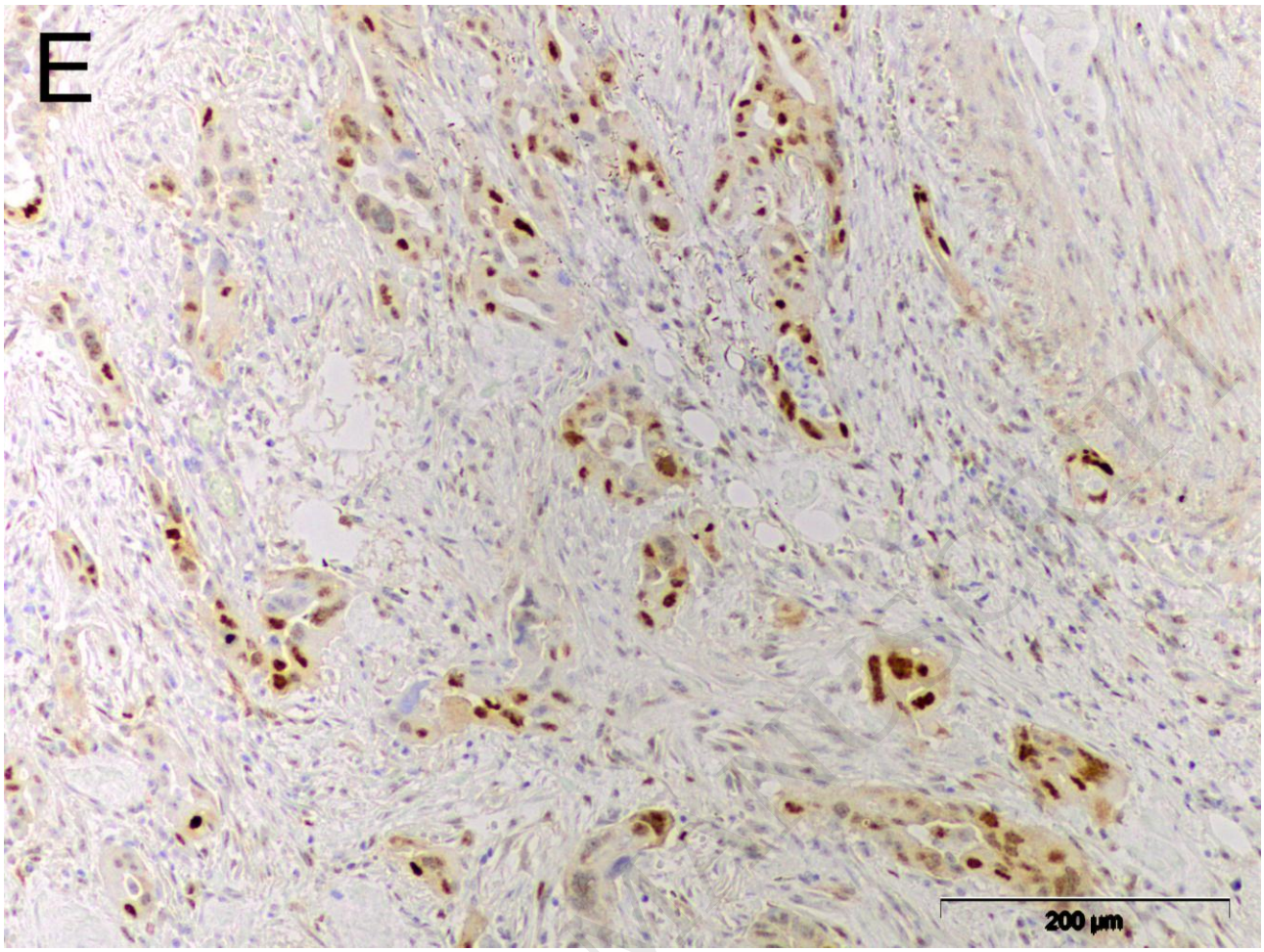
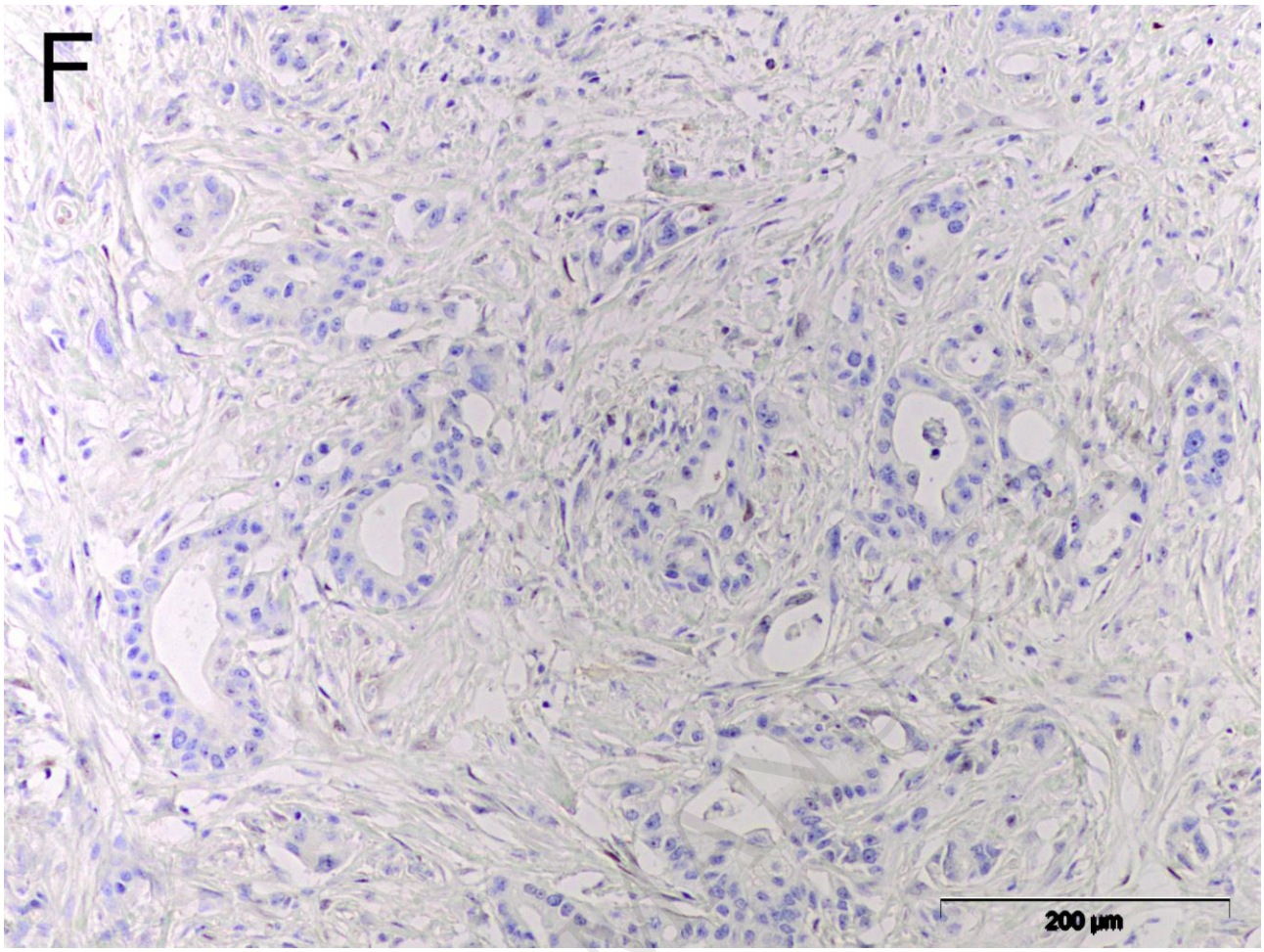


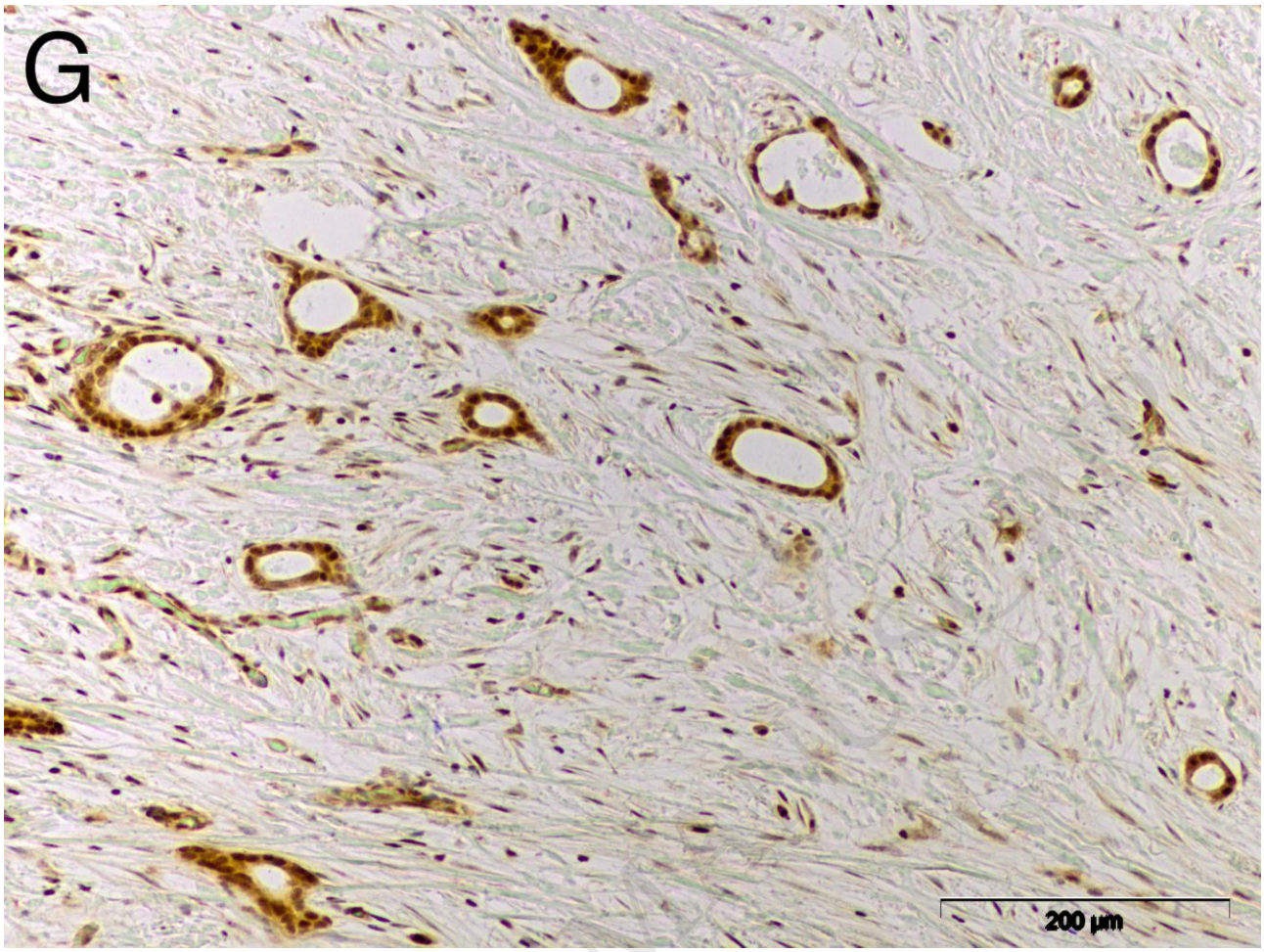
Fig 2

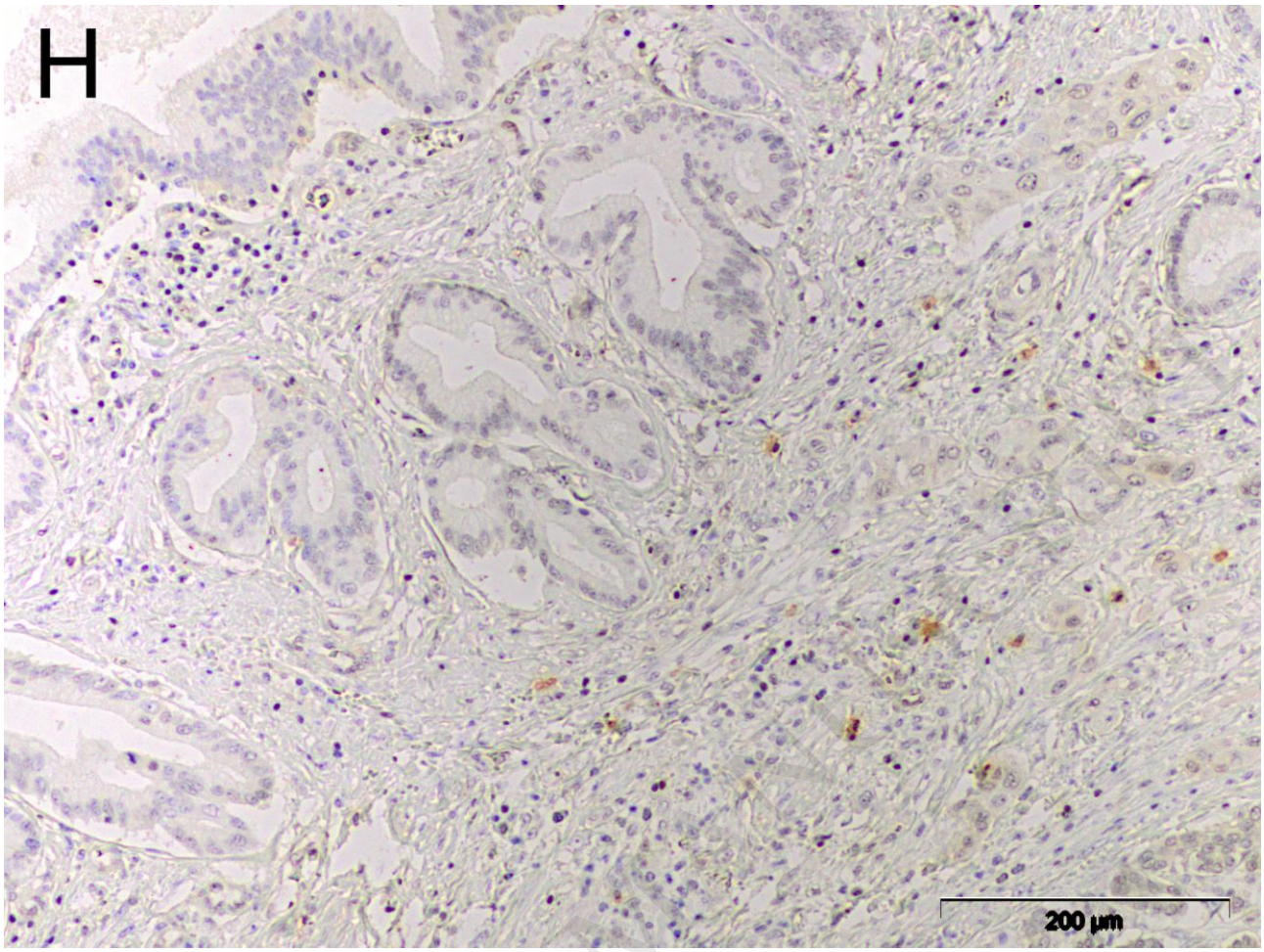


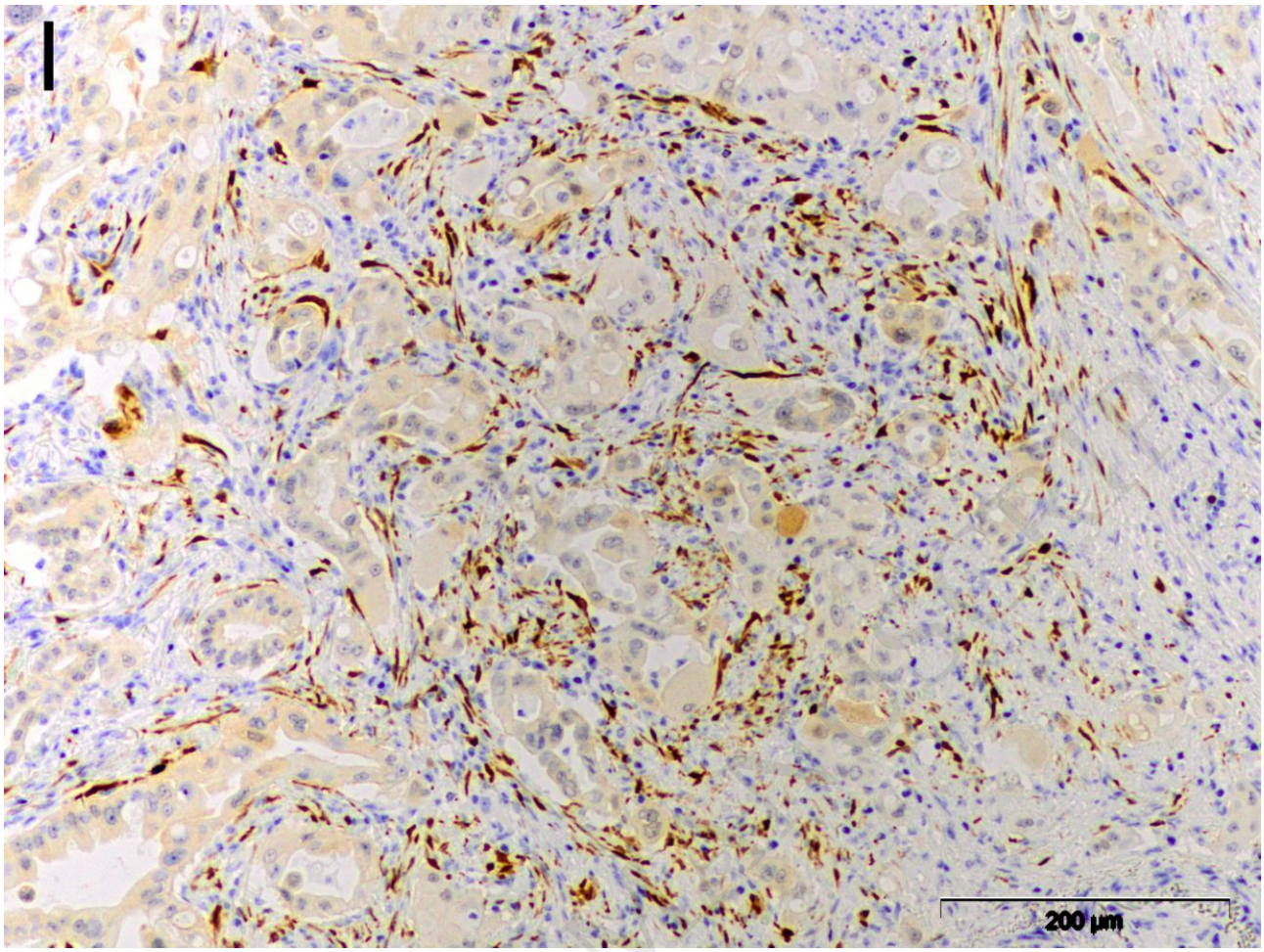












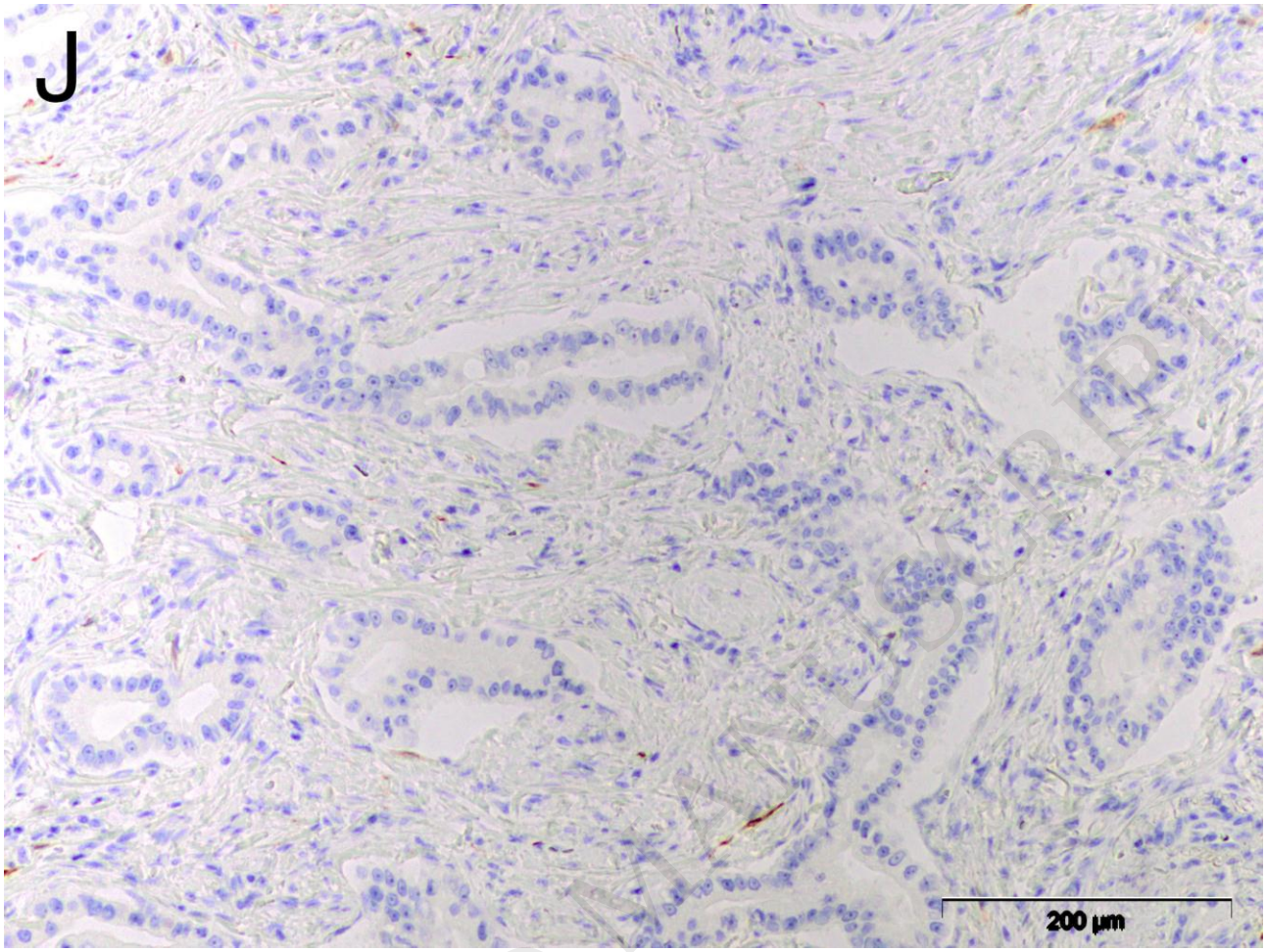


Table 1. Patient characteristics

Characteristic	***N (%)***
Gender	
Male	52 (51%)
Female	49 (48%)
Not available	1 (1%)
Age at diagnosis	
<50 years	6 (5.9%)
50-59 years	29 (28.4%)
60-69 years	31 (30.4%)
>69 years	29 (28.4%)
Not available	7 (6.9%)
Tumour (T)	
1	6 (5.9%)
2	28 (27.5%)
3	57 (55.9%)
4	8 (7.8%)
Not available	3 (2.9%)
Nodal metastasis (N)	
No	39 (38.2%)
Yes	61 (59.8%)
Not available	2 (2.0%)
Distant metastasis at the time of diagnosis (M)	

No	90 (88.2%)
Yes	10 (9.8%)
Not available	2 (2.0%)
Distant metastasis during follow-up	
No	83 (81.4%)
Yes	19 (18.6%)
Not available	0 (0.0%)
Grade	
I	25 (24.5%)
II	42 (41.2%)
III	27 (26.5%)
Not available	8 (7.8%)
Local relapse	
No	64 (62.7%)
Yes	35 (34.3%)
Not available	3 (2.9%)
Type of surgery	
Palliative	5 (4.9%)
Whipple	83 (81.4%)
Other, with curative intention	14 (13.7%)

Table 2. Immunohistochemical methods

Primary antibody	*Manufacturer of the primary antibody*	*Dilution*	*Immunostaining method*
p16ink4 (Ref9511)	Ventana Medical System, Inc., Tucson, USA	1:4	Dako REAL™ EnVision™ Detection System (Dako Denmark A/S, Glostrup, Denmark)
CDK4 (NBP1-31308)	Novus Biologicals, Littleton, USA	1:100	Dako REAL™ EnVision™ Detection System (Dako Denmark A/S, Glostrup, Denmark)
Rb1 (HPA050082)	Atlas Antibodies Ab, Bromma, Sweden	1:500	Dako REAL™ EnVision™ Detection System (Dako Denmark A/S, Glostrup, Denmark)
FEN1 (ab109132)	Abcam, Cambridge, UK	1:1000	Novolink Polymer Detection Systems (Leica Biosystems, Newcastle, UK)
MGMT1 (ab108630)	Abcam, Cambridge, UK	1:750	Dako REAL™ EnVision™ Detection System (Dako Denmark A/S, Glostrup, Denmark)

CDK4, Cyclin-dependent kinase 4

Rb1, Retinoblastoma-associated protein-1

FEN1, Flap endonuclease-1

MGMT, O-6-methylguanine-DNA methyltransferase

Table 3. Percentages of evaluable cases showing expression of p16ink4, CDK4, Rb1, FEN1 and MGMT.

	Adenocarcinoma cells	*Benign exocrine pancreatic tissue*

	Nucleus (%)	Cytoplasm (%)	Nucleus (%)	Cytoplasm (%)
p16ink4	43.3	44.9	33.3	33.3
CDK4	55.0	17.0	0.0	0.0
Rb1	75.8	13.1	1.2	0.0
FEN1	100.0	67.6	100.0	59.0
MGMT	82.0	79.0	79.4	65.7

CDK4, Cyclin-dependent kinase 4

Rb1, Retinoblastoma-associated protein-1

FEN1, Flap endonuclease-1

MGMT, O-6-methylguanine-DNA methyltransferase

Table 4. Protein expression showing independent prognostic value in multivariate analysis.

Protein	*Immunostaining location (nuclear/cytoplasmic/other)*	*Endpoint*	*Subgroup*	*Cox multivariate analysis: Risk ratio*	*95 % CI*	*Variables included in Cox multivariate analysis*	*Kaplan Meier univariate analysis: p-value*	*Median survival in low expression group (months)*	*Median survival in high expression group (months)*
CDK4	Nuclear, cancer cells	DFS	T3-4	2.148	1.081-4.272	CDK4, N	0.029	18	7
CDK4	Islets of Langerhans, benign cells	RFS	-	2.874	1.261-6.550	CDK4, T, N	0.012	64	17
MGMT	Nuclear, cancer cells	OS	N1	2.148	1.066-4.329	MGMT, T	0.032	19	12
MGMT	Nuclear, cancer cells	DFS	N1	2.114	1.019-4.385	MGMT, T	0.044	16	9
MGMT	Nuclear, cancer cells	RFS	N1	9.028	2.192-37.179	MGMT, T	0.002	28	14
MGMT	Nuclear, cancer cells	OS	T3-4	1.878	0.962-3.667	MGMT, N	0.065	19	12
FEN1	TLS	DFS	-	2.619	1.132-6.059	FEN1, T, N	0.025	18	6
FEN1	TLS	RFS	-	3.758	1.148-12.299	FEN1, T, N	0.029	32	17

CDK4, Cyclin-dependent kinase 4

MGMT, O-6-methylguanine-DNA methyltransferase

FEN1, Flap endonuclease-1

TLS, Tertiary lymphoid structure

OS, Overall survival

DFS, Disease-free survival

RFS, Relapse-free survival