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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 = .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 = .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 regulator also have predictive significance, but further studies are required to evaluate this.

1. 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

Abbreviations: Rb, retinoblastoma-associated protein-1; CDK4/6, cyclin-dependent kinases 4 and 6; MGMT, O6-methylguanine-DNA-alkyltranferase; FEN1, flap endonuclease-1; PDAC, pancreatic ductal adenocarcinoma; DFS, disease-free survival; RFS, relapse-free survival; OS, overall survival; TLS, tertiary lymphoid structures

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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.

2. Materials and methods

2.1. 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.

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%)

2.2. 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 min for Rb1 and 12 min 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 min. After that, the samples were cooled at room temperature for 20 min. 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 min. The samples were incubated with primary antibodies (Table 2) at +4 °C for 30 min for p16 and CDK4 staining, for 60 min 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.

2.3. 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