Familial Melanoma and Pancreatic Cancer: studies on genotype, phenotype and surveillance

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Application of a serum protein signature for pancreatic cancer to separate cases from controls in a pancreatic surveillance cohort

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ABSTRACT

BACKGROUND

Pancreatic cancer (PC) surveillance is currently offered to individuals with a genetic predisposition to PC, but routinely used radiological screening modalities are not entirely reliable in detecting early-stage PC or its precursor lesions. We recently identified a discriminating PC biomarker signature in a sporadic patient cohort. In this study, we investigated if protein profiling can accurately distinguish PC from non-PC in a pancreatic surveillance cohort of genetically predisposed individuals.

METHODS

Serum samples of 66 individuals with a *CDKN2A* germline mutation who participated in the pancreatic surveillance program (5 cases, 61 controls) were obtained following a standardized protocol. After sample clean-up, peptide and protein profiles were obtained on an ultrahigh resolution MALDI-FTICR mass spectrometry (MS) platform. A discriminant score for each sample was calculated with a previously designed prediction rule, and the median discriminant scores of cases and controls were compared. Individuals with precursor lesions of PC (n=4) and individuals with a recent diagnosis of melanoma (n=4) were also separately considered.

RESULTS

Cases had a higher median discriminant score than controls (0.26 vs 0.016; p=0.001). The only individual with pathologically confirmed precursor lesions of PC could also be clearly distinguished from controls, and having a (recent) medical history of melanoma did not influence the protein signatures.

CONCLUSIONS

Peptide and protein signatures are able to accurately distinguish PC cases from controls in a pancreatic surveillance setting. MS-based protein profiling therefore seems to be a promising candidate for implementation in the pancreatic surveillance program as an additional screening modality.

INTRODUCTION

Pancreatic cancer (PC) is one of the most lethal cancers with a 5-year survival rate of only 5%.[1] The first clinical symptoms generally appear relatively late when the tumour is already in an advanced stage. To improve prognosis, PC has to be detected at an earlier stage in which curative surgical resection is still possible. Therefore, in the last decade, pancreatic surveillance programs for high-risk individuals have been set up, aimed at detecting early-stage PC or relevant precursor lesions in individuals with a genetic predisposition to PC.[2]

At the Leiden University Medical Center (LUMC), such a pancreatic surveillance program was initiated in the year 2000 for individuals with a *CDKN2A* germline mutation.[3] These individuals have a familial predisposition for developing cutaneous melanoma, a condition known as Familial Atypical Multiple Mole Melanoma (FAMMM) syndrome, but also a 15-20% lifetime risk for developing PC.[4] Because many individuals with a specific Dutch founder mutation in the *CDKN2A* gene (a 19bp deletion known as p16-*Leiden*) are living in the vicinity of Leiden, a relatively large cohort of these patients is under pancreatic surveillance in the LUMC. The surveillance program consists of annual abdominal magnetic resonance imaging (MRI and MRCP) and optionally endoscopic ultrasound (EUS). Although these screening modalities are generally able to detect early-stage PC or relevant precursor lesions of PC, the diagnostic yield of surveillance programs using these modalities varies greatly and only a subset of patients with a screen-detected PC have an early-stage cancer.[2,5] Therefore, there is a need to improve the current pancreatic surveillance program.

One way to improve PC surveillance programs is to use serum biomarkers as an additional non-invasive screening modality.[6-9] These biomarkers have to discriminate cancer patients from non-cancer patients or even patients with precursor lesions of PC. Currently, only the mucin-associated carbohydrate antigen CA 19-9 is routinely used, but has not proven to be an adequate biomarker for detecting early-stage PC.[10] Many studies have been published on novel individual biomarkers for the early detection of PC, but none of them have been implemented in daily practice so far.[11,12]

In our center, a discriminating PC biomarker signature was recently identified by following a serum peptide and protein profiling strategy based on a combination of automated single-step sample clean-up and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS).[13,14] The most detailed protein signatures were obtained using an ultrahigh resolution MALDI Fourier transform ion cyclotron resonance (FTICR) MS

platform that provided case-control classifications with a sensitivity and specificity both well above 85%.[15] A discriminating prediction rule was validated for this classification. The methodology used in our previous studies is graphically displayed in *figure 1* (left-hand side). Based on these encouraging results it was concluded that such protein signatures are a promising candidate for implementation in the current pancreatic surveillance program as an additional screening modality. The aim of the current study is therefore to determine whether ultrahigh resolution protein profiling (using MALDI-FTICR-MS) in serum can accurately distinguish individuals with PC from non-PC in a novel cohort of *CDKN2A* mutation carriers enrolled in the pancreatic surveillance program, using the previously designed and validated prediction rule for the classification of individual samples (*figure 1*, right-hand side).

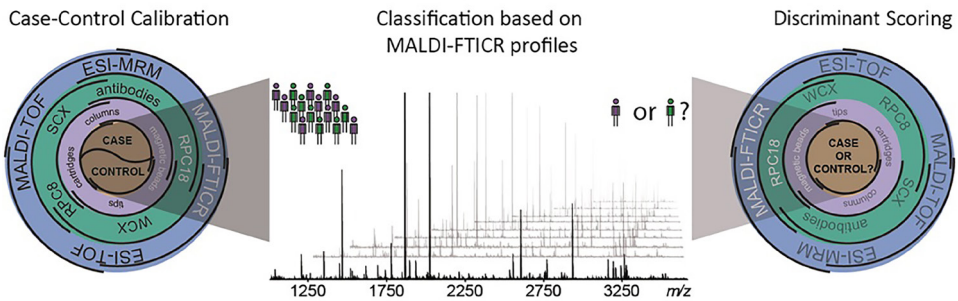


FIGURE 1: Serum peptide and protein profiling strategy, aiming for patient classification based on matrix-assisted laser desorption/ionization (MALDI) Fourier transform ion cyclotron resonance (FTICR) mass spectrometry.

Various peptide and protein signatures have been reported based on a single-step sample clean-up procedure using a combination of a carrier (depicted in the inner shell) with capture material (depicted in the middle shell), and a mass spectrometer (depicted in the outer shell). Previously, our group has reported signatures for PC based on weak-cation exchange (WCX) with MALDI time-of-flight (TOF) [Velstra et al. 2013], and reversed-phase (RP) C18 with MALDI-TOF [Velstra et al. 2015]. In the current study an ultrahigh resolution RPC18-MALDI-FTICR signature is used that was obtained in a case-control calibration and validation design (left-hand side) [Nicolardi et al. 2015]. Serum samples from *CDKN2A* mutation carriers are analysed in an identical way to obtain a discriminant score (right-hand side).

PATIENTS AND METHODS

PATIENT COHORT AND BLOOD SAMPLING

Individuals with a *CDKN2A* germline mutation who participate in the pancreatic surveillance program at the Leiden University Medical Center were eligible for inclusion. A complete medical history was obtained at the start of surveillance, including a medical history of melanoma or other cancers. Subsequently, annual MRI and MRCP with optionally EUS was performed and in case of an abnormal finding, either close follow-up with MRI/MRCP and EUS or surgery was advised by a multidisciplinary team, as previously described.[3] Any cancer occurring in follow up was registered. Cases were defined as having a pathologically confirmed diagnosis of PC. Controls were not diagnosed with PC, and included individuals with relevant precursor lesions of PC. These were defined as either pathologically proven precursor lesions (intraductal papillary mucinous neoplasm (IPMN) and pancreatic intraepithelial neoplasia (PanIN) [16]), or radiological cystic lesions ≥ 5 mm suspicious for IPMN.

Serum samples from the cases with PC were obtained prior to surgery. Serum samples from the controls without PC were obtained during their annual surveillance visit at the outpatient gastroenterology clinic. Only one sample was collected per individual. Samples were collected over a time period ranging from April 2008 until January 2015. Additional serum samples of *CDKN2A* mutation carriers with PC who did not participate in the surveillance program were available through an on-going research project of the Department of Surgery, in which serum samples of all patients with PC are obtained prior to surgery. Samples were collected and processed following a standardized high-throughput clean-up protocol as previously described.[17,18] Informed consent was obtained from all individuals, and the study was approved by the Ethics Committee of the Leiden University Medical Center (#P03.147).

SAMPLE PROCESSING AND MALDI-FTICR MASS SPECTROMETRY PEPTIDE PROFILING

The isolation of peptides and protein from serum was performed using a fully automated, high-throughput protocol based on solid-phase extraction (SPE) with RPC18-funtionalized magnetic beads, as previously described.[15,18] Subsequently, MALDI-profiles were obtained on a MALDI-FTICR platform that allows mass analysis of serum peptides and proteins with isotopic resolution up to 15,000 Da. A detailed description of this approach and workflow, as well as the subsequent data processing, was previously described by Nicolardi *et al*.[15] For this study, only so-called low-mass (LM) data (i.e., up to *m/z*-value 4000) was used for statistical analysis. The serum samples were blindly analysed.

STATISTICAL ANALYSIS

Our group previously designed a prediction rule to classify a serum sample as either case or control using logistic regression ridge shrinkage (LRRS) analysis.[15,19] By applying the same prediction rule to the LM data acquired in this study, a "discriminant score" was calculated for each sample. Samples were grouped according to their known disease status and the median discriminant scores per group were compared using a Mann-Whitney-Wilcoxon test. Individuals with precursor lesions and individuals with a recent diagnosis of melanoma were also separately considered.

RESULTS

PATIENTS

A total of 66 individuals (42 females, 64%) were included in the study. Sixty-one individuals had a molecularly proven *CDKN2A* germline mutation, of which 60 had the p16-*Leiden* mutation (c.225_243del19; RefSeq NM_000077.4). One individual carried the c.67G>C mutation, which is also associated with PC [not published data]. The remaining 5 individuals had a medical history of melanoma (or PC, #4 *table 2*), and a close relative with a proven *CDKN2A* germline mutation, which makes them highly likely of being a carrier. Patient characteristics are shown in *table 1*. Five individuals (all female) had PC, with a mean age of 54 years (range 39-62 years). Two of five cases had a medical history of melanoma, but no other cancers occurred in the case group. The remaining 61 individuals (37 females, 61%) had no PC. The mean age of the control group was 53 years (range 42-72 years). Thirty-eight controls had a medical history of melanoma, and a few other cancers occurred in the control group (see *table 1*). One individual in the control group had a melanoma 1 month prior to serum sampling (#2 *table 3*), and one individual had a melanoma 1 month after serum sampling. Two other individuals had cancer ≤12 months before or after serum sampling (both melanoma; 12 months prior and 9 months after). These melanomas were non-metastatic.

Detailed information about the case group is shown in *table 2*. Three cases were participating in the surveillance program, of which two were diagnosed with PC at the first screening round (prevalent) and one was diagnosed on a subsequent screening round (incident). This latter individual (#1, *table 2*) had a normal MRI two years earlier but missed her MRI a year later. She was diagnosed with a 3.6 cm tumour in the subsequent year. Two of five cases were not participating in the surveillance program, and had their serum drawn prior to surgery as part of standard (research) procedure at the Department of Surgery.

TABLE 1. Patient characteristics

Diagnosis	No. of Patients	Age (range)	M:F	Medical History of Melanoma (of which multiple)	Medical History of Other Cancers (No. of Individuals) *
PC	5	54 (39-62)	0:5	2 (1)	None
No PC	61	53 (42-74)	24:37	38 (12)	SCC of larynx (1) † SCC of mouth (1) † SCC of skin (1) BCC of skin (3) Phyllodes sarcoma of breast (1)
With precursor lesions	*4/61*	*54 (45-63)*	*2:2*	*3 (1)*	None
Total	66	53 (39-74)	24:42	40 (13)	As above

SCC = Squamous Cell Carcinoma, BCC = Basal Cell Carcinoma
** None of these cancers occurred within a year prior to serum sampling*
† These cancers occurred synchronously in one individual

TABLE 2. Tumour Characteristics of Cases with PC

	Age	M/F	Medical History of Cancer	Mode of Diagnosis	Loc.	Tumour Size (cm)	Tumour Stage (TNM)	Tumour Grade
1	57	F	-	Surveillance, incident	Tail	3.6	T2N0M0 (Stage IB)	2
2	62	F	Me 56 yrs	Surveillance, prevalent	Head-corpus	0.5	T1N0M0 (Stage IA)	1
3	62	F	Me 31 yrs (2x)	Symptomatic	Head	5.0	T3N1M0 (Stage IIB)	2
4	39	F	-	Symptomatic	Proc. uncinatus	1.5	T3N1M0 (Stage IIB)	n/a
5	47	F	-	Surveillance, prevalent	Corpus	5.7	T3N1M0 (Stage IIB)	3

Me = Melanoma

Four individuals in the control group had relevant precursor lesions of PC, of which detailed information is shown in *table 3*. All four individuals had cystic lesions ≥ 5 mm suspicious for IPMN, but only one individual had a surgical resection due to growth of the lesion. Pathological examination of the resected pancreas of this patient confirmed the presence of an IPMN lesion, as well as multifocal PanIN1-2 lesions.

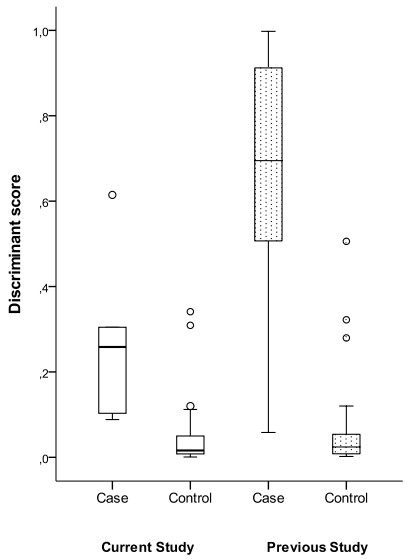
TABLE 3. Precursor Lesions of PC in the Control Group

	Age	M/F	Medical History of Cancer	Findings Pancreatic Surveillance	Surgical Intervention	Pathology
1	63	F	-	Multicystic lesion of 15 mm in head-corpus region, stable for 6 years and growth to 17 mm in the 7th year. Suspicious for BD-IPMN. Two cystic lesions (8 mm, head and 5 mm, tail), stable for 2 years. Suspicious for BD-IPMN	Subtotal pancreatectomy	BD-IPMN; Multifocal PanIN1–2
2	59	M	>15 Me from age 27, most recent at age 59	Multicystic lesion of 7 mm in proc. uncinatus, suspicious for BD-IPMN, stable for 2 years	Not performed	n/a
3	45	F	Me 42 yrs	Cystic lesion of 7 mm and multicystic lesion of 7 mm in head region, both suspicious for BD-IPMN, stable for 2 years	Not performed	n/a
4	49	M	Me 44 yrs	Cystic lesion of 13 mm in corpus-tail region, suspicious for BD-IPMN, stable for 2 years	Not performed	n/a

Me = Melanoma, BD-IPMN = Branch duct intraductal papillary mucinous neoplasm, PanIN = Pancreatic intraepithelial neoplasia

Statistical classification of serum profiles

High-quality MALDI-FTICR data was obtained from all samples and therefore all samples were suitable for further statistical analysis. In *figure 2*, boxplots of the calculated discriminant scores for cases (n=5) and controls (n=61) are shown. Boxplots of the data from our previous study are displayed in *figure 2* as well. Cases from our previous study had a noticeable higher score than cases from the current study, as can be seen in *figure 2*. This can probably be explained by the fact that more cases in our previous study had metastatic (lymph nodes positive or distant) disease, i.e. stage IIB or higher (83% compared to 60% in the current study). As was shown in our previous study, a more advanced tumour stage is associated with a higher discriminant score. The difference could further be caused by a systematic re-calibration effect. Nonetheless, the boxplots show that cases with PC are accurately distinguished from controls without PC in the new surveillance data. The median discriminant score for cases is 0.26 and for controls 0.016, which differs significantly (p value 0.001 using the Mann-Whitney-Wilcoxon test).



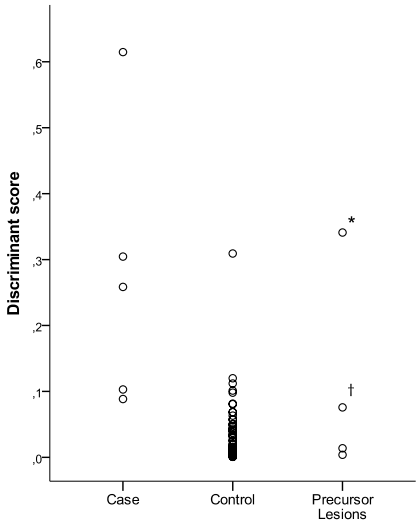


FIGURE 2.

FIGURE 3.

FIGURE 2: Boxplots of the discriminant scores for cases and controls of the current study and of our previous study

The boxplots on the left represent the data of the current study. For comparison, boxplots of the data from our previous study [Nicolardi et al. 2015] are displayed on the right. The generally higher discriminant scores of cases in the previous study compared to cases in the current study can probably be explained by the fact that more cases in our previous study had metastatic (lymph nodes positive or distant) disease, i.e. stage IIB or higher (83% compared to 60% in the current study). A more advanced tumour stage is associated with a higher discriminant score. A systematic re-calibration effect could further explain the difference.

O = Outliers

FIGURE 3: Scatter plot of the discriminant scores of the current study; individuals with precursor lesions are separated from controls

This figure shows all the individual discriminant scores of the 66 included individuals, subdivided in cases (n=5), controls (n=57) and individuals with precursor lesions (n=4).

** Individual #1 (table 3); discriminant score of 0.34, † Individual #2 (table 3); discriminant score of 0.08*

Scores of individuals with precursor lesions of PC are separately shown in *figure* 3. The only individual with pathologically proven precursor lesions of PC (#1 in *table 3*, * in *figure 3*) had a relatively high score of 0.34, well above the median score of controls and comparable with the scores of the cases. The individual with precursor lesions as well as a melanoma 1 month prior to serum sampling (#2 in *table 3*, † in *figure 3*) had a score of 0.08 and scored above the 75th percentile of the median score of the control group. The other two individuals with (radiological) precursor lesions had a score below the median of the control group. Apart from individual #2 (*table 3*), there were three other individuals with a melanoma diagnosed shorty before or after serum sampling. These individuals had a score near or well below the median score of the control group.

DISCUSSION

In this study, we analysed biomarker profiles in a pancreatic surveillance cohort of *CDKN2A* mutation carriers with and without PC using the same methodology as in our earlier work. By applying the previously designed prediction rule for the classification of serum samples, cases with PC could be accurately distinguished from controls without PC. Also, individuals with suspicious precursor lesions of PC might be distinguished from controls, and having a (medical history of) melanoma probably does not influence the protein signatures. Protein profiling therefore has potential to be included in the pancreatic surveillance program, where it, as an addition to current screening methods, can aid in the decision whether a patient will need surgery or not.

Different biomarkers have been extensively studied in sporadic patient cohorts over the last decades,[12,20,21] but this is the first study to investigate the role of biomarkers in a pancreatic surveillance cohort of genetically predisposed individuals. Recent studies from the University of Marburg did however investigate biomarkers in familial PC (FPC) individuals with PC or relevant precursor lesions of PC in a non-surveillance setting.[22,23] Interestingly, the (few) individuals with pathologically confirmed high-grade precursor lesions (PanIN2-3) in their studies had significantly elevated serum biomarker levels prior to surgery and the levels dropped to the normal range after surgery. FPC individuals having relevant precursor lesions of PC could thus accurately be distinguished from healthy controls using their proposed biomarker sets, and the authors argued that biomarkers may be suitable for the early detection of precursor lesions of PC in high-risk individuals.

Indeed, a major goal of screening is the detection of precursor lesions of PC,[2] and their prevalence in *CDKN2A* mutation carriers is evident. Vasen *et al* reported that 11% of *CDKN2A*

carriers in the surveillance program had possible precursor lesions (*ductectasias*) on radiology.[3] Potjer *et al* reported an even higher number (16%), and concluded that precursor lesions might have a high malignant potential in *CDKN2A* carriers, compared to precursor lesions in FPC individuals.[24] In order to be implemented in a pancreatic surveillance cohort, it is therefore important that potential serum biomarkers not only distinguish non-cancer patients from cancer patients, but also from patients with relevant precursor lesions of PC. In this study, there was only one patient with histologically confirmed precursor lesions (IPMN and PanIN1-2), and as mentioned those precursor lesions, especially the IPMN, might have a relatively high malignant potential because the patient was a *CDKN2A* mutation carrier. This patient had a protein signature comparable to those with PC. The other three patients with less suspicious precursor lesions on radiology had a normal to near-normal protein profile. Therefore, it seems likely that patients with substantial precursor lesions might be accurately distinguished from healthy *CDKN2A* carriers using serum protein profiling, although numbers are too small to make definite conclusions.

A second requirement for biomarkers to be implemented in a pancreatic surveillance cohort of high-risk individuals, especially *CDKN2A* carriers, is that the signatures are not disturbed by the occurrence of other types of cancer. The FAMMM syndrome (due to a *CDKN2A* germline mutation) is mainly characterized by a very high risk (70%) of developing cutaneous melanoma, and 62% of the carriers in this study indeed had a medical history of melanoma. Having a medical history of melanoma did not influence the protein signatures in general, as cases could still accurately be distinguished from controls in this cohort. Also, the four controls with a recent diagnosis of melanoma did not evidently diverge from the other control patients. Only the individual with both a recent diagnosis of melanoma and radiological precursor lesions had a slightly higher discriminant score than the other controls, but that could be caused by the presence of precursor lesions as argued above. In addition to the high risk of developing melanoma and PC, *CDKN2A* mutation carriers also have a higher risk of developing head and neck squamous cell carcinoma, which emphasises that FAMMM syndrome is a true tumour syndrome.[25,26] It is therefore also important to know if these cancers influence the protein signatures, but that could not be investigated in the current study due to the fact that there was no recent diagnosis of this type of cancer in the study group. There was only one individual in this cohort with two synchronous tumours of the larynx and mouth 4 years prior to serum sampling, without recurrence after treatment and a very low discriminant score.

The most important limitation of this study is sample size. More individuals with PC and, preferably, histologically confirmed high-grade precursor lesions are needed to investigate if these individuals definitely can be distinguished from healthy *CDKN2A* individuals. These

patients are however very rare and it would take years to collect only a few more patients. Also, more patients with other tumours than PC at or around the time of serum sampling are needed in order to investigate if those tumours intervene with the protein signatures. A second limitation is that we did not collect samples after surgical treatment, and therefore we could not investigate if the high discriminant scores declined after surgery. Future implementation of protein profiling in the surveillance program, with standardized yearly serum sampling, including post-surgery sampling, will ensure more patients with different types of cancer or precursor lesions of PC.

Since current screening strategies for PC are not entirely reliable for detecting early-stage PC or its (high-grade) precursor lesions, there is a strong need to improve the pancreatic surveillance program. As is shown in this preliminary study, protein profiling seems a very promising method to be included as an additional non-invasive screening modality. Previously, similar MS-based profiling studies in our group provided promising results with regard to peptide and protein signatures for the early detection of breast cancer and colorectal cancer,[18,27] and thus protein profiling seems suitable for cancer surveillance in general.

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