Integrated Project „Novel molecular diagnostic tools for the prevention and diagnosis of pancreatic cancer (MolDiag-Paca)"

Executive Summary

2nd reporting period; Aug 1st 2007 – July 31st 2008

Malignant tumours of the pancreas, known as pancreatic carcinomas, remain among the most serious challenges in modern medicine. There are currently no means for the reliable diagnosis of early stages and for curative treatment of late stages of the tumour.
The overall aim of this EU Framework Programme 6 Integrated Project ("MolDiagPaca"; visit our project website at www.moldiagpaca.eu) is to make use of genetic profiles of pancreatic cancer and precursor lesions to improve the outcome of pancreatic cancer patients by providing novel and highly efficient molecular diagnostic tools for an early diagnosis.

During the 2nd annual period of our project (months 13-24), the consortium ambitiously continued to work towards the final project goals (development of molecular tools for early diagnosis of pancreatic cancer). Collection of resources (i.e. tissue, serum, plasma, urine, DNA, RNA, tissue arrays) continued, databases and registries were updated and extended, candidate genes and proteins were more closely investigated as well as protocols optimised, and established assays were validated.

Over the past year and with great use for workpackage (WP) 1 the European Registry for Hereditary Pancreatitis and Familial Pancreatic Cancer (EUROPAC) was extended from 445 to 461 families with pancreatitis and from 208 to 287 families with more than one case of pancreatic cancer (P2). Since in these families the causative mutation is most often unknown, SNP based association analysis on 58 of these families is being pursued. The data has already been generated, however analysis using family based association test is still underway. The putative proto-oncogene palladin was further analysed by P2. However, no mutations were found in the individuals tested.
Furthermore, a case-control study with 2000 cases and controls each has been devised within WP1 to identify polymorphisms associated with pancreatic cancer both independently or by

interacting with environmental factors. For this purpose, P5/P22 have designed a computerised questionnaire that will be used to personally interview patients by trained monitors. P2 has developed a Bayesian model for inclusion of risk factors.

Already during the first 12 months of the project, a relational pancreatic expression database was generated by P3 (http://www.pancreasexpression.org/). Over the second annual period of the project this database has continuously been updated with new data from all available sources including novel data provided by other European Partners. Thus it will continue to represent the most comprehensive resource for pancreatic cancer researchers. For the consortium the database greatly facilitates the selection of individual target genes or signatures suitable to develop molecular diagnostic and prognostic tools for an early diagnosis of pancreatic cancer.

Expression analysis of rare exocrine tumours (Ampulla of Vater Cancer (AVC), Acinar Cell Carcinoma (ACC), Solid Papillary Tumors (SPT), Intraductal Papillary Mucinous Tumours (IPMT)) have been performed (P7). In summary, expression profiling has already allowed differentiation of tumour types, identified genes which may serve as biological markers to assess cancer aggressiveness and/or enable biological subclassification.

For the profiling of endocrine tumours 32 PET samples were analysed and genomic aberrations in each sample were identified. The copy number data from PETs were compared with our published PDAC data. Unsupervised hierarchical cluster analysis has demonstrated that genomic profiling is highly distinctive in these tumours. The current analysis aims to identify the significant correlation between specific genetic alterations and clinical features of these tumours.

P1 has continued the analysis of pancreatic tissues using the dedicated diagnostic cDNA array. The focus of the current phase of the project is on establishing one-step multiclass diagnosis of different entities of malignant and benign tumours of the pancreatico-biliary system. Due to the high clinical relevance, priority has been given to the accurate differentiation of tumour types commonly causing obstruction of the distant bile duct.

Following the establishment of a dedicated DNA-microarray on glass with 7500 tumour-associated genes, P4 has continued the profiling of pancreatic tissues with the goal of identifying novel potential target genes for RNA-based diagnostic assays. To date, a total of 100 samples have been processed and analyzed.

P8 has identified a number of miRNAs which are differentially overexpressed in pancreatic carcinoma. Thus a miRNA specific qRT-PCR assay for microdissected pancreatic tissues (acinar and ductal cells, and different progression stages: PanIN-1-3 and PDAC) is being developed. To this end two different qRT-PCR assays have been established and as a proof of concept, miRNA 196a was shown to be over-expressed only in more advanced PanIN stages and in nearly 100% of PDACs. Currently additional miRNAs are under study and the collection of microdissected tissues is extended.

Another objective of the project is to apply advanced proteomic analyses to detect and identify proteomics-based diagnostic biomarkers of pancreatic cancer in serum, pancreatic juice and urine (P2 & P3). Serum samples from pancreatic cancer, chronic pancreatitis, bile duct obstruction and healthy control were analysed using both 4-plex and 8-plex iTRAQ labels. Moreover, quantitative data were obtained on depleted serum samples using iTRAQ. Specifically, 14 proteins displayed altered levels in the cancer serum pool compared to other groups, including some proteins previously shown to be involved in carcinoma. Two novel proteins identified in this study are currently undergoing further validation.

Another approach on the protein level is the identification of pancreatic juice protein biomarkers by mass spectrometry methods. After initial difficulties, the protocol for pancreatic juice protein analysis is now running. The LC-MS-MS analysis is in full progress and data will be available shortly.

Proteomic analysis of urine specimens derived from patients with chronic pancreatitis (CP), pancreatic adenocarcinoma (PDAC) and healthy controls has successfully been performed using 2D-DIGE technology. Although validation has proved challenging due to posttranslational modifications and processing of proteins in urine, our study for the first time clearly demonstrates that urine is a valid source of non-invasive biomarkers in patients with pancreatic diseases.

The enrichment of exosomes from conditioned media has been established for a number of pancreatic carcinoma cell lines. Preparations are characterized for the expression of exosomal marker proteins by quantitative Western blotting. Work on proteomic characterization is ongoing; preliminary results suggest that the comparison of spectral counts found for individual proteins in the secretome and the exosome fractions provides comprehensive insight into the composition of both subproteomes.

Epigenetic analysis, especially the characterisation of pancreatic cancer-specific changes in DNA methylation (P4) remains being a major focus of the consortium. Software packages and computational means are in place to deal with the data and to identify relevant variations and associations by tested algorithms and statistical processes. Currently, we have about 250 samples ready to be studied on the various platforms (P4, P17) with more samples being prepared continuously. A set made from 50 patients is complete and data analysis is in progress.

Figure: Example of epigenetic variations in a particular region. Identified by chip analysis, the results were confirmed by bisulfite sequencing. Nine repetitions were done for each sample. Solid circles represent methylated cytosines, open circles stand for unmethylated

A great hope for significant advancements in the early diagnosis of pancreatic ductal adenocarcinoma (PDAC) and its precursior lesions (PanINs) lies in the development of novel molecular imaging approaches based on the *in vivo* detection of cancer-specific single molecular markers identified in high-throughput approaches. In a highly coordinated target selection process two targets were given the status of "validated targets" and were thus prioritized for imaging tool development (P1, P4, P8, P10, P13, P15, P19, P20).

Five additional targets were given the status of "first line candidate". These candidates are still under investigation, mainly due to technical difficulties in validating the targets via immunohistochemistry.

P15 installed and established a new and the first pan-European free space fluorescence mediated tomographical system: Three different cell lines were chosen to establish pancreatic cancer xenografts. Cells were injected subcutaneously into SCID mice to monitor tumor growth. FMT imaging will be performed with target-specific tracers provided by P19. A library of substrates against one of the target proteins was designed and synthesized. The most selective substrate, which is 7050 times more active than any published substrate, was selected from the library and labelled with a NIR-dye for imaging.

P20 concentrated their efforts on the development of suitable imaging markers for PET and SPECT for three additional target proteins. The first generation of radiolabeled markers potentially applicable for PET and SPECT were synthesized for all three targets. Their relevance was confirmed in *in vitro* experiments in pancreas tumour cell lines.

Since the last report, the UK competent authority (the MHRA) has begun the process of enforcing EU Directives 2001/20/EC and 2005/28/EC as passed into UK law as statutory instruments SI 1031, 1928, 2984, 2031. In order to continue to work with trial samples

obtained as part of WP7, P2 has established Good Clinical Laboratory Practice (GCLP) laboratories at site in Liverpool.
Recruitment for screening trials now stands at 69 familial pancreatic cancer patients and 24 hereditary pancreatitis patients. Markers from WP4 (P2) and WP5 (P2) have been tested in samples from these patients. No cancers have been detected in these patients to date.
Ethical approval (MREC) for the obtaining additional (follow-up) samples from patients has been completed. Separate ethical approval for prospective collection of ESPAC-4 patients has been approved. Out of a total of 2154 samples identified we have centralised 251 paraffin blocks (which are being used to prepare tissue microarrays) and 125 frozen samples. The relatively low number of samples collected to date reflects the complex ethical aspects of exporting trial samples, however, most sites are now at an advanced stage in overcoming these hurdles and are committed to sending samples. P12 has made antibodies to key enzymes involved in gemcitabine metabolism available to MolDiagPaca and these antibodies are being piloted for use in IHC by P2.

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