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Gene Expression Based Categorization of Pancreas Transplant Biopsies

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Abstract

Gene expression profiling has emerged as a powerful strategy to define transcriptional mechanism activated in diseased human organs. We performed a feasibility study of mRNA based pancreas transplant biopsy stratification.

The mRNAs expression of 32 genes observed in renal transplant dysfunction and ten pancreas specific genes were evaluated in 26 pancreas transplant biopsy specimens by qRT-PCR using TaqMan Low Density Array (TLDA) technology.

Unsupervised 2D hierarchical clustering segregated the biopsies in two main cluster branches, A and B. Six out of seven patients (85.7%) in cluster A and 6 out of 19 (31.6%) in cluster B retained functioning pancreas allograft. CD20/MS4A1 mRNA and protein were detected in 4 biopsy specimens in cluster B. Three of the four CD20 positive pancreas transplant were subsequently lost despite anti-rejection treatment.

This feasibility study demonstrates the association of pancreas gene expression with clinical outcome of the transplant and justifies the evaluation in further studies.

Pancreas transplantation is one of the accepted treatment modalities for a selected group of type 1 diabetic patients, particularly if they also have end stage renal disease (ESRD) necessitating renal transplantation [1, 2]. Over the years, the outcome of pancreas transplant has improved [3, 4]. However, beside early peri-operative graft loss, the rejection, both acute and chronic, remains the major cause of pancreas graft loss. The diagnosis is challenging and response to treatment variable [5, 6]. Pancreas biopsy is the only mean to make histological diagnosis and to guide therapeutic intervention [5, 7]. A key limitation of conventional histology is its' descriptive nature, giving limited insight into pathogenic mechanism.

Studies evaluating molecular markers associated with kidney transplant dysfunction have provided mechanistic insight and additional levels of diagnostic information [8–10]. There

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are no studies available to date to describe the molecular alterations in pancreas transplant dysfunction in humans.

We hypothesized that the immunological mechanisms underlying pancreas transplant rejection are similar to those involved in kidney transplant rejection process, but organ-specific gene expression markers for endocrine and exocrine pancreas function has to be established.

In this pilot study, we characterized the gene expression patterns of transplant pancreas biopsy specimens and tested their association with the clinical characteristics of the patients.

We retrospectively identified 26 archived transplant pancreas biopsy specimens obtained for the clinical suspicion of pancreas allograft dysfunction over the time period between 1999 and 2006. Four specimens from normal portions of human tumor pancreatectomies were used as control tissue. The study was approved by the Institutional Review Board.

All biopsy specimens were processed for routine light microscopy: immediate fixation in 4 % PBS-buffered formaldehyde followed by paraffin-embedding and storage at room temperature.

Histological diagnosis used the Maryland classification [7] in a blinded manner by two pathologists experienced in assessing pancreas transplant biopsies.

The mRNA was extracted from the formaldehyde-fixed, paraffin-embedded (FFPE) biopsy samples according to the methodology described previously [11].

Thirty-two mRNA markers involved in the rejection processes of kidney transplants, six pancreas function-specific mRNAs (Insulin, Glucagon, Somatostatin, Amylase, Cholecystokinin A receptor, Elastase 2A), four pancreas re-generational transcription factors (Ipf1, Maf, Maf-B, Neuro-D1), and five housekeeping genes (POLR2A, PPIA, UBC, 18s rRNA, GAPDH) were studied for the quantitative analysis of the mRNA expression values (supplementary table 2).

The qRT-PCR was performed using TaqMan® Low Density Array (TLDA) technology on a TaqMan ABI 7900 Sequence Detection System (Applied Biosystems). The cDNA expression values were compared following the 2^{-Ct} technique [12]. The expression of target genes was normalized to a calibrator, the mean of the reference genes. Real-time RT-PCR expression values were analyzed with the dChip software (<http://www.biostat.harvard.edu/complab/dchip/>). The software computes a distance based unsupervised hierarchical cluster [13] for genes as well as for samples. The clustering parameters are set to the standard values of 1- r for distance and to centroid linkage where r denotes the Pearson correlation coefficient. The cluster visualization color-codes the standardized and centered expression values to pure green for an expression of -3 or lower, black for mean expression and pure red for an expression of 3 or higher.

For CD20 protein detection, immunohistochemistry method was employed with the use of L26 mouse monoclonal antibody against CD20 antigen, the avidin-biotin conjugated with

horseradish peroxidase, diaminobenzidine as substrate and counterstained with hematoxylin (Ventana Medical System, Tuscon, AZ).

The demographic characteristics of 26 patients are shown in table 1. There were 14 simultaneous pancreas and kidney transplants (SPK) and 12 pancreas after kidney transplants (PAK). Average age at the time of pancreas transplant was 37.8 ± 5.8 years. Induction was used in 96.2% (25/26) of pancreas transplant recipients. Maintenance immunosuppression consisted in triple-drug regimen in 96.2% of patients (25/26). Tacrolimus (Tac) accounted for 20 and cyclosporine A (CsA) for 5 patients. All patients received maintenance doses of steroids. 92% of subjects (24/26) were on mycophenolate mofetil. The median time from transplant to biopsy was 3.1 months (0.4, 105.4). The most common reasons for biopsy were elevation of pancreatic enzymes (15/26, 57.7%) and hyperglycemia (9/26, 34.6%). Twenty-two pancreas biopsy specimens (22/26, 84.6%) showed various degrees of acute rejection according to the Maryland classification. Two had chronic rejection. Two showed normal histology. One had histological picture resembling graft versus host disease (GVHD) lesions.

Sufficient RNA for qRT-PCR analysis was extracted from all 26 biopsy specimens. qRT-PCR expression values of 32 gene markers, 10 pancreas specific and 5 reference mRNAs were detected in at least two samples assayed by the TLDA. To evaluate the relationship of the mRNA expression signatures to each other and to group the patients according to their mRNA fingerprints, unsupervised cluster analysis was performed. Patient samples were sorted in the horizontal cluster dendrogram into two main groups, cluster A and cluster B (Figure 1). Cluster A included a group of 7 biopsies and 4 samples of normal pancreas tissue with expression of pancreas functional and regenerative markers. Cluster B included 19 samples with shared up-regulated expression of genes known as markers for acute (AR) or chronic rejection (CR). Within cluster B two additional sub-clusters could be defined: B1 (12 samples) expressed mainly genes induced in acute kidney transplant rejection. B2 (7 samples) showed an enrichment of the CR marker.

All 10 pancreas-specific mRNA markers showed co-regulation in the lower cluster group (vertical dendrogram), and were associated with repression of in cluster B biopsies. 27 of 32 mRNA markers of acute, chronic or acute and chronic rejection were co-regulated in the upper cluster (vertical dendrogram) with induction in cluster B biopsies (Fig.1).

We compared pancreas survival following biopsy as a clinical outcome variable between the two molecular defined subgroups. Six of 7 patients (85.7%) from cluster A maintained pancreas allograft function with a mean follow-up length of 53.9 months (± 36.3 months) while only 6 of 19 in cluster B (31.6%) had preserved pancreas allograft function at 29.0 months post-transplant (± 27.1 months). The cluster B specimens were associated with a less favorable clinical outcome (χ^2 , $p = 0.014$).

CD20/MS4A1 mRNA expression was detected in a subgroup of biopsies within the B2 sub-cluster, being present in 4 out of 7 of these pancreas biopsies (Figure 2a). Immunohistochemical staining of the respective biopsy sections confirmed the various

degree of CD 20 positive staining in all 4 specimens (Fig. 2b and 2c). Three of them had lost function during subsequent follow-up despite intensified immunosuppressive therapy.

To the best of our knowledge, the pilot study presented here is the first attempt aimed to delineate the molecular markers and pathways involved in pancreas transplant rejection process taking advantage of information gained from related studies in the field of renal transplantation [8, 10, 14, 15]. A qRT-PCR based technology was able to detect gene expression profile using RNA extracted from archived pancreas biopsy specimens. Unsupervised 2D hierarchical clustering patterns segregated biopsy specimens into groups that suggested an association with clinical outcome. Extension of this approach to more comprehensive cohorts of patients is essential to explore molecular predictors and molecular pathways involved in the process of pancreas transplant dysfunction.

Molecular mechanisms of immune activation have been extensively studied and documented in renal transplant literatures over the past decade [8, 9, 14–16]. Limited data exist in pancreas transplants literatures [17, 18]. Rejection, acute and chronic, continues to be the most common cause of graft failure in technically successful pancreas transplants [4, 6]. Treatment of acute rejection is often empirical and the response often not uniform and predictable [5, 19]. Recently, two groups of investigator have reported isolated cases of pancreas transplant rejection mediated through production of alloantibody that responded to humoral, but not cellular mediated therapeutic intervention [20, 21]. The presence of CD20 signals (mRNA transcript and protein) in some of pancreas biopsy specimens supports the possible involvement of a humoral mechanism in certain cases of pancreas rejection and the need for further refinement in therapeutic intervention.

Limitations of the current study is related to its' retrospective nature and small sample size. As the cases were collected over the past decade, there have been several changes in the institutional immunosuppression protocols, both induction and maintenance. In addition, the management of acute rejection was based on individual assessments of each particular patient. This significantly limits the conclusions to be drawn concerning the correlation of molecular expression pattern and clinical outcome. The main aim of this pilot study was to document the technical feasibility of molecular analysis in archived pancreas transplant biopsy specimens and the association between clinical parameters and molecular profiles. The detection of CD20 infiltrates in pancreas transplants can serve as an example for the potential novel insight to be gained by this approach.

In conclusion, we documented various degrees of immune activation, cellular and possibly humoral, and concomitant loss of endocrine and exocrine mRNA markers of pancreas function in archived pancreas biopsy specimens. This study gives first evidence of the feasibility of mRNA based stratification of pancreas transplant failure and provides thereby justification for mRNA marker analysis in large cohorts of archived biopsy materials to test the diagnostic power of molecular profiling in pancreas transplants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Cluster dendrogram of mRNAs differentially regulated in human pancreas tissue. Induction of mRNA is shown in red, repression in green.

Horizontal dendrogram sorts patients according to similarity in expression profiles of mRNAs.

Vertical dendrogram sorts mRNAs according to similarity in expression changes between patients.

Two main groups of patients are separated, defined as cluster A and B.

mRNA markers were selected according to association with acute, chronic or acute and chronic rejection (AR, CR, AR+CR) of kidney transplants and pancreas function (PAN) or

pancreas regeneration (PAN_REG). For details concerning mRNA evaluated see table 2 in supplement.

Figure 2.

a: CD20/MS4A1 mRNA expression levels (ratio to reference gene panel) in 4 control pancreas and 26 transplant biopsies.

b–c: Immunohistochemistry for CD20 protein expression in pancreas biopsy specimens:

b: Biopsy with CD20/MS4A1 mRNA up-regulation;

c: Biopsy without CD20/MS4A1 mRNA up-regulation.

Table

Demographic and clinical characteristics

Samples	Race	Gender	Age	Transplant type	Induction*	IS regimen	Biopsy time (mo.)	Indication	Maryland classification	Treatment	Graft failure
1	AA	M	31	SPK	Thymo	Tac/M/P	0.6	Hyperglycemia	III	OKT3	No
2	C	M	50	SPK	Basiliximab	Tac/M/P	0.7	Hyperglycemia	0	None	No
3	C	M	35	SPK	Basiliximab	Tac/M/P	2.0	Hyperglycemia	I	Steroids	No
4	AA	M	35	SPK	Thymo	Tac/M/P	44.6	Elevated enzymes	II	Steroids	No
5	C	M	39	SPK	OKT3	CsA/M/P	67.5	Elevated enzymes	0	None	No
6	C	M	34	SPK	Basiliximab	CsA/M/P	35.3	Elevated enzymes	II	Thymo	No
7	C	F	40	PAK	Thymo	Tac/M/P	4.9	Elevated enzymes	II	Thymo	Yes
8	C	M	38	PAK	Basiliximab	Tac/M/P	5.1	Elevated enzymes	III	None	Yes
9	C	F	45	PAK	Basiliximab	CsA/M/P	3.0	Elevated enzymes	II	Steroids	No
10	C	F	46	SPK	OKT3	S/P	105.4	Elevated enzymes	III	Steroids	Yes
11	C	M	40	SPK	Basiliximab	Tac/M/P	0.6	N/A	IV	Thymo	Yes
12	C	M	34	PAK	Basiliximab	CsA/M/P	10.8	Elevated enzymes	GVHD	None	Yes
13	C	M	28	PAK	OKT3	Tac/M/P	3.1	Elevated enzymes	IV	Steroids	Yes
14	C	F	30	SPK	Basiliximab	Tac/M/P	20.3	Elevated enzymes	II	Steroids	No
15	AA	F	35	SPK	Thymo	Tac/M/P	3.4	Elevated enzymes	IV	None	Yes
16	C	M	34	SPK	Basiliximab	Tac/M/P	1.1	Elevated enzymes	I	None	No
17	C	M	48	PAK	Basiliximab	Tac/M/P	0.9	Hyperglycemia	II	Thymo	No
18	C	F	34	SPK	Basiliximab	Tac/M/P	0.4	Hyperglycemia	II	Steroids	Yes
19	C	F	36	PAK	Basiliximab	Tac/M/P	0.8	Elevated enzymes	IV	Steroids	Yes
20	C	M	39	PAK	Basiliximab	Tac/M/P	0.6	Elevated enzymes	II	None	No
21	C	M	27	SPK	None	Tac/M/P	10.1	Elevated enzymes	III	OKT3	Yes
22	C	F	37	PAK	Basiliximab	Tac/M/P	0.6	Hyperglycemia	III	Thymo	Yes
23	AA	M	34	PAK	Basiliximab	Tac/M/P	0.4	Hyperglycemia	III	OKT3	Yes
24	C	M	40	SPK	Basiliximab	Tac/M/P	1.6	N/A	III	Steroids	No
25	C	M	35	PAK	Basiliximab	Tac/M/P	33.9	Hyperglycemia	IV/CR	None	Yes
26	C	F	32	PAK	OKT3	CsA/M/P	73.1	Hyperglycemia	CR	None	Yes