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5-gene differential expression predicts stability of human intestinal allografts

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ABSTRACT

In intestinal allografts, endoscopy and histology detect the injury once changes in the bowel wall architecture have occurred. We aimed to identify a molecular signature that could predict early deterioration, within histologically indistinguishable biopsies with "minimal changes" (MC) pathology. Sixty biopsies from 12 adult recipients were longitudinally taken during 8 years post-transplant. They were classified as either stable (STA) or non-stable (NSTA) according to the prospectively recorded number, frequency and severity of rejection events of the allograft. In a discovery set of MC samples analyzed by RNA-Seq, 816 genes were differentially expressed in STA *vs* NSTA biopsies. A group of 5 genes (ADH1C, SLC39A4, CYP4F2, OPTN and PDZK1) correctly classified all NSTA biopsies in the discovery set and all STA biopsies from an independent set. These results were validated by qPCR in a new group of MC biopsies. Based on a logistic regression model, a cutoff of 0.28 predicted the probability of being a NSTA biopsy with 85% sensitivity and 69% specificity. In conclusion, by analyzing MC samples early after transplantation, the expression of a 5-gene set may predict the evolution of the bowel allograft. This prognostic biomarker may be of help to personalize care of the intestinal transplant recipient.

1. Introduction

Intestinal transplantation (IT) is a procedure to treat irreversible intestinal failure (caused in most cases by short bowel syndrome) in patients suffering life-threatening complications of parenteral nutrition. Until 2014, nearly 2500 ITx cases had been performed throughout the world, but despite recent improvements, management of IT recipients is still challenging (Grant et al., 2015; Kubal et al., 2015; Smith et al., 2017; Sudan, 2014). In contrast to other organs, the transplanted small bowel bears an important proportion of immune cells and commensal bacterial flora, making it prone to inflammation and infection. The acute rejection rate of intestinal allografts is higher than in other organs and may appear early after transplantation and repeatedly with time (Ruiz, 2012). Severe rejection episodes involve immune mechanisms that eventually damage the intestinal absorptive capacity and favor the translocation of luminal bacteria, which are closely associated with graft loss and patient death. Other post-transplant (post-Tx) events such as lymphoproliferative disease, graft *versus* host disease, ulcers, enteritis, eosinophilic syndromes or *de novo* autoimmunity, derive from a complex interplay of both non-immune and immune factors as yet barely understood (Fishbein, 2009; Kroemer et al., 2016; Loo et al., 2017; Ranganathan et al., 2015; Selvaggi et al., 2007).

Abbreviations: AUC, area under the curve; CI, confidence interval; cpm, counts per million; DEGs, differencial expressed genes; FDR, false discovery rate; GLM, generalized linear model; IRX, indeterminate for rejection; IT, intestinal transplantation; MC, minimal changes; MDS, multidimensional scaling; NSC, nearest shrunken centroids; NSTA, non-stable; qPCR, quantitative polymerase chain reaction; RF, random forest; RNA-Seq, RNA sequencing; RX, rejection; SBT, small bowel transplantation; STA, stable; SVM, support vector machine; Tx, transplant

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Monitoring of IT integrity relies on endoscopic and histological examination of intestinal mucosa biopsies, continuously performed during the first post-Tx period. However, in a recent series investigating 1770 endoscopies in pediatric transplants, 45% of biopsy-proven rejections showed a normal gross appearance, suggesting a low correlation between endoscopy and histology (Yeh et al., 2015). Thus, in many cases, these procedures detect the problems once tissular changes have occurred, although the responsible molecular mechanisms may significantly precede them.

In the clinical transplantation arena there is a need for predictive biomarkers for allograft evolution. The transcriptome analysis of the transplanted organ was introduced to identify molecular signatures for rejection, tolerance or drug toxicity. These studies aim to translate into the clinical setting by proposing the measurement of a reduced number of differentially expressed genes to anticipate the event and guide therapy. However, most studies show two important limitations. Firstly, because they analyze very selected and distant pathology phenotypes, *i.e.* normal *vs* acute rejection biopsies, their results do not provide additional information to that already known. Secondly, in all cases gene expression is measured with probe arrays which do not interrogate the whole transcriptome but an *a priori* determined set of transcripts.

In the present work, we aim to identify a molecular signature of IT deterioration before it translates into visible histological injuries. We explored histologically indistinguishable IT biopsies with minimal changes (MC), which corresponded to either stable (STA) or non-stable (NSTA) intestinal transplants. Because of its potential to quantify the complete transcriptome we used massive RNA sequencing (RNA-Seq) to measure differently expressed genes (Marguerat and Bahler, 2010; Wang et al., 2009). With the unprecedented use of this tool for biomarker discovery in transplantation, we identified and validated a 5-gene set as a molecular classifier with a good predictive capacity for the evolution of IT. The measurement of these 5 genes in IT biopsies by quantitative PCR (qPCR) may identify unstable intestinal allografts and guide early therapeutic intervention.

2. Materials and methods

2.1. Patients, biopsies and study design

The study included 12 adult recipients who had undergone small bowel (SBT) or multivisceral transplantation between 2005 and 2015 in our hospital (Table 1). Biopsies from ileal mucosa were taken at 10–20 cm from ileostomy. Endoscopic and biopsy controls were performed soon after transplantation and at progressively increasing intervals (2 per week during 1st month, 1 per week during 2nd and 3rd months, 1 every two weeks during 4th and 5th months, 1 monthly until the end of the first year, and then once every six months). According to this protocol, biopsies were routinely taken even in the absence of clinical symptoms, and additional endoscopies and biopsies were performed when clinical events appeared. Data from histological diagnosis from paired biopsies (examined by at least two independent pathologists) and immunosuppressive therapy were also collected. MC was considered in biopsies without specific histopathologic changes, closely resembling the features of normal gut. Indeterminate for rejection (IRX) was considered in biopsies with minimal rejection, < 6 apoptosis in 10 crypts, mild inflammatory infiltrate and edema. Rejection grade 1 (RX) was defined by increased apoptosis in crypts and epithelium, occasional endothelitis and mild villous blunting (Fig. S1) (Andreev et al., 2011; Wu et al., 2003).

Table 1
Clinical data from small bowel and multivisceral recipients.

Patient	Underlying disease	Gender	Age at Tx	Organ Tx
P1	Familial Adenomatous Polyposis	Male	40	SBT
P2	Familial Adenomatous Polyposis	Male	44	SBT
P3	Familial Adenomatous Polyposis	Male	24	SBT
P4	Acute mesenteric ischemia	Female	38	SBT
P5	Crohn's disease	Male	38	SBT
P6	Acute mesenteric ischemia	Male	64	SBT
P7	Acute mesenteric ischemia	Male	45	SBT
P8	Politraumatism	Male	30	SBT
P9	Gastrointestinal stromal tumor (GIST)	Female	47	SBT
P10	Familial Adenomatous Polyposis	Female	32	MVT
P11	Primary intestinal lymphangiectasia Haemolytic uremic syndrome	Female	26	SBT + Kidney
P12	Intestinal pseudo-obstruction syndrome	Female	30	MVT

Tx: transplantation/SBT: small bowel transplantation/MVT: multivisceral abdominal transplantation.

All patients received alemtuzumab for induction therapy, except P11 (SBT plus kidney recipient) and P12 who received thymoglobulin. The main immunosuppressive drug was tacrolimus in all patients. Mycophenolate mofetil was added in patients P1, P2, P3, P4 and P12 within the first three months post-Tx. P4, P6, P10, P11 and P12 received everolimus at 2 months, 6 years, 4 years, 8 months and 1 month post-Tx respectively. Corticosteroids were administered because of a rejection episode in P3, P4, P7, P8, P10 and P11 and because of Crohn's disease (P5), adrenal insufficiency (P10) or kidney allograft (P11).

A total of 60 IT biopsies were longitudinally taken between 2011 and 2015 in a post-Tx period of 13 to 3101 days. Fifty biopsies showed MC, 7 were IRX, and 3 showed RX (Table 2). All 50 MC biopsies were classified into two groups. STA group included all biopsies from allografts of patients who had experienced no rejection, and biopsies from patients who rejected, obtained at least 15 days after rejection, if no other event occurred within the next six months. When rejection occurs, 3 boluses of metilprednisolone are administered followed by decreasing doses of prednisone for 5–10 days. By day 15 after the last rejection biopsy, corticosteroids are minimized and patients are considered clinically stable. NSTA group included biopsies obtained between rejection episodes (those occurring < 6 months apart), together with biopsies collected within 15 days before the first rejection episode. By defining the biopsies under these criteria we hypothesized that biopsies in the STA category may represent quiescent intestines whereas biopsies in the NSTA group may represent intestinal allografts prone to suffer rejection events. This categorization was established prior to analysis.

Fig. 1A shows the workflow for biomarker identification and validation. Twenty-four MC samples (discovery and test sets, 17 STA and 7 NSTA) from 8 patients (Fig. 1B and Table 2) were analyzed by RNA-Seq. Eighteen biopsies for the discovery set (11 STA and 7 NSTA) were obtained from 4 IT recipients. To minimize differences due to the intrinsic characteristics of patients, 3 recipients (P2, P3 and P4) were included because they were the only ones with both STA and NSTA biopsies. All biopsies from the fourth patient included in the discovery set (P1) were STA samples, but they were obtained at a post-Tx period matching that of the other 3 recipients (most biopsies taken within the first year post-Tx) (Fig. 1B). The other 6 biopsies from the remaining 4 patients (P5, P6, P7 and P8) were used as the test set. These 6 biopsies were all STA and were taken at long post-Tx time (more than two years) (Fig. 1B and Table 2). Genes obtained in the RNA-Seq stage as possible biomarkers were validated as predictors by qPCR and logistic regression in a validation set formed by 26 independent biopsies (17 STA and 9 NSTA) from 9 patients. The model was then tested in a rejection set formed by 10 new biopsies (7 IRX and 3 RX) from 4 patients, and in 16 biopsies from the initial discovery set (Fig. 1 and Table 2).

Experiments were approved by the institutional review board (CEIC 13/370) and written informed consent was obtained from all patients.