



Samenvatting proefschrift J. Yang

“Molecular and genetic markers for the prediction of kidney transplant outcome”

Promotie: 19 december 2018
Leids Universitair Medisch Centrum Leiden

Promotor:
Prof. dr. F.H.J. Claas

Copromotor:
Dr. M. Eikmans

Summary and general discussion

Kidney transplantation is the preferred treatment of patients with end stage renal disease, as it provides longer patient survival and better quality of life compared to dialysis. Prediction of DGF, response to treatment of acute rejection, and long-term graft outcome remain difficult when using merely clinical parameters. Numerous studies have reported on the predictive value of molecular markers for AR and adverse graft outcome. However, the heterogeneity of AR and the variation among transplant centers lead to opposing results and preclude a general clinical application. In the first part of this thesis, we aimed to investigate the molecular markers of steroid resistance and long-term graft survival on the basis of acute rejection biopsies. In the second part, we focused on genetic variants associated with acute rejection in kidney transplantation. Previously, expression of S100 calcium binding proteins A8 and A9 was found to be related to favourable transplant outcome. In the final part of this thesis we found evidence for immune regulatory effects of these S100 proteins.

Selection of SYBR green master mix

Quantitative polymerase chain reaction (qPCR) is a sensitive and specific technique based on SYBR green chemistry to measure gene expression levels. The type of PCR device and master mix used may affect the accuracy and reliability of the gene expression assays. Three commercial SYBR green PCR mixes (ABI, Bio Rad, Roche) were tested for 79 specific primer pairs, each targeting an immune-related transcript. We found that most primer sets (N=66, 94.3%) generated a single sharp melting peak with all tested PCR mixes, in case the prescribed PCR protocol was strictly followed. The use of ABI mixes, compared to the other mixes, led to lower Cq values (higher expression signal) for cDNA and lower background levels for negative control DNA samples. The type of PCR device had a smaller influence on the results than the source of SYBR green mix. Based on the data obtained in these studies, we decided



to measure all molecular transcripts in biopsy samples using the ABI mix on Vii7 PCR equipment.

Relation of pre-transplant gene expression levels with the type of donor

In transplantations with a kidney from a deceased donor, none of the markers investigated in pre-implementation biopsies was predictive for DGF, which is in contrast with a previous study showing that the BAX:BCL2 ratio was elevated in a patient group suffering from DGF (19). We found that expression of C2 and C3, and BAX:BCL2 ratio was higher in kidneys from deceased donors compared to living donors, indicating a role of the complement and apoptosis pathways in ischemia reperfusion injury. This observation confirmed previous studies showing that complement components are significantly higher in deceased donors compared to living donors (20), and that apoptotic cell death is initiated as reflected by an increased BAX and decreased BCL2 during normothermic ischemia injury (21). Therefore, inhibition of complement and apoptosis pathways may act as therapeutic target to protect from the effects of IRI.

Alterations in gene expression are the result of inflammatory cell infiltration

Gene expression alterations between paired pre-implantation and acute rejection biopsies were analyzed in 75 patients. The majority of TLRs (TLR1, -2, and -3, and TLR6, -7, -8, -9, and -10), C2, and BAX:BCL2 mRNA levels were increased, whereas the expression levels of C4 and the complement regulators (CD46, CD55, and CD59) were decreased at the moment of AR compared to the situation before implantation. The changes in expression levels of TLR4, TLR5, C3, and CR1 varied among the patients with acute rejection. Immunohistochemical staining for TLR4, TLR9, and BCL2 confirmed that their expression was relative higher in acute rejection group than in patients with stable graft function. We speculate that the changes in mRNA expression are the result of infiltration of inflammatory cells. Therefore, the correlation between the expression level of innate immunity genes and inflammatory markers (CD163, CD68, CD20, CD3e) at time of AR was analyzed. The expression of TLR1, TLR4, TLR6-10, C2, C3, CR1, and BAX:BCL2 was positively correlated with one or more inflammatory markers, while the expression of CD46 and CD59 was negatively correlated with macrophage markers. The influx of inflammatory cells can at least partly explain the altered gene expression between implantation and AR biopsies. However, most of the genes, expressed during AR, show no association with any of the Banff classification scores.

Risk assessment of the occurrence of steroid resistance rejection: the E-score

Steroid resistant acute rejection is associated with inferior long-term graft outcome (10, 36). Prediction of steroid resistance during acute rejection would open the possibility to treat patients immediately with the optimal immunosuppression and prevent unreparable nephron damage during the period of steroid therapy. In chapter 4 we found that an increased mRNA expression of endothelial-epithelial related genes at the moment of acute rejection predicts the responsiveness to steroid therapy.



Endothelial cells line the interior surface of glomeruli and peritubular capillaries and they mediate crucial inflammatory processes. The endothelium-epithelium transcript profile, including TM4SF18, PGM5, and CD34, which are involved in angiogenesis and cell-cell adhesion, may reflect the integrity of the nephrons and the capacity of cells to repair tissue injury. This may explain why the decreased expression profile is associated with resistance of steroid treatment. In line with our finding, several studies showed that severe intimal arteritis and destruction of microvasculature predict steroid resistance of the rejection (49-51). Therefore, the endothelium-epithelium expression profiles may provide novel markers to predict steroid resistant rejection.

Prediction of long-term graft survival

In chapter 3 we showed that patients with high TLR4 expression during acute rejection have inferior graft survival compared to patients with low TLR4 expression. TLR4 in the allograft may bind to intracellular ligands released by dead cells, and provide additional proinflammatory signaling to enhance inflammation. Although the antirejection therapy successfully normalized kidney graft function, as reflected by decreased serum creatinine, the high expression of TLR4 may lead to production of higher levels of proinflammatory cytokines and chemokines that induce inflammatory cells infiltration into the allograft after the antirejection therapy and contribute to chronic allograft nephropathy.

A high Bax:Bcl2 ratio during AR predicts inferior long-term graft survival in patients who had received a transplant from a deceased donor, and it may reflect a high extent of apoptosis in the transplant. Apoptosis of parenchymal cells may directly lead to the loss of kidney function. However, apoptotic cells attract phagocytic cells into the graft and may be rapidly cleared (53, 54). The accumulated phagocytic cells can be triggered by a danger signal and mediate chronic allograft nephropathy (55). If the apoptotic cells in allograft are not rapidly cleared, they undergo necrosis and release damage-associated molecular patterns (DAMPs) that initiate immune responses (56). Thus, monitoring of the Bax:Bcl2 ratio during AR may offer a predictive value with respect to long-term graft survival. Future studies should contain a more in-depth analysis of the presence and kinetics of dying cells in the graft, and their possible impact on outcome.

Genetic risk factors in kidney transplant: small effect and lack of validation

In chapter 5 we performed a GWAS of acute rejection in kidney transplantation. The significant candidate SNPs identified by current GWAS could not be verified in an independent cohort from another transplant center. In line with a previous study, we found that patients with acute rejection show a higher C allele frequency for the rs1801274 SNP in FCGR2 compared to the stable graft function group (64). Apart from this specific SNP, the majority of previously published SNPs could not be confirmed in the current GWAS. Consistent with a well powered GWAS in bone marrow transplantation, the previously reported genetic variants were most likely false positive findings (65). As discussed in chapter 7, the main limitation of GWAS is the requirement of stringent significance thresholds ($P < 5 \times 10^{-8}$). Only SNPs with a big effect on transplant outcome could be captured in our relatively small-sized GWAS.



Thus, any false positive findings in our study may result from the small effect of individual genetic variants. Individual SNPs identified by GWAS usually have a small effect by themselves on a complex trait such as acute rejection, explaining only less than 10% of susceptibility to the disease, even when all available genetic variants are combined (66). To identify true positive single SNPs, which have a small effect, and to overcome the issue of validation, the only way to increase sample size is by (inter)national collaboration in the field of kidney transplantation.

The role of non-HLA antigens has increasingly been reported in kidney transplantation (67-69). As shown in chapter 6, we found no effect of genomic missense SNP mismatching on kidney transplant outcome. Besides, the mismatch load, reflecting the total amount of mismatching of SNPs in coding sequences between recipient and donor, does not have any effect on AR or long-term graft function. If the mismatch load had any effect, living related transplantations with lower mismatch load should have lower incidence of rejection and longer allograft survival compared to living unrelated transplantations that have higher mismatch load. However, a recent meta-analysis showed there was no difference between living related and unrelated kidney transplantations in acute rejection and graft survival rates (70). A reason for the negative finding may be that the effect of mismatching of missense SNPs is low, under the condition of HLA mismatching and efficient immunosuppressive therapy.

The immune regulatory effect of S100 calcium binding proteins A8 and A9

Relatively high tissue expression of S100A8 and S100A9 during AR is associated with a beneficial effect on long-term kidney graft survival (71, 72). We found in chapter 8 that most of S100A9+ cells co-expressed CD68 and HLA-DR, but only one-third of them expressed CD163. This suggests that S100A9 represents a marker for a distinct macrophage population infiltrating the graft. We further found that S100A9 expression varies greatly among CD14 positive myeloid cells. Unfortunately we were not able to sort monocytes based on their expression level of S100A9 using SmartFlare RNA detection probes. Cytokine expression profiles between S100A9^{high} and S100A9^{low} subsets were not significantly different. We did find that overexpression of S100A8/A9 in monocyte-derived macrophages leads to increased reactive oxygen species (ROS) production, as well as to increased IL-10 mRNA expression. The extracellular ROS may have a negative impact on T cell activation and their subsequent proliferation (73), which may dampen the immune response in the allograft. The consistent increase of IL-10 may represent an anti-inflammatory mediator in such immune responses, even though the protein level of IL-10 could not be detected in the supernatant of the transfected cells. We hypothesize that the anti-inflammatory effect of S100A8/A9 proteins explains their beneficial effect on kidney graft survival.

Conclusions

The results presented in this thesis demonstrate that several molecular and genetic markers are associated with kidney transplant outcome. We showed that a decreased expression profile of endothelial-epithelial cells during AR is associated with resistance to steroid therapy, suggesting that endothelial cell integrity is involved in the efficacy of antirejection treatment. The elevated TLR4 expression and BAX:BCL2 ratio during AR



independently predict inferior long-term graft survival. Previous studies have shown that relatively high expression levels of S100A8/A9 at time of acute kidney transplant rejection are associated with beneficial long-term transplant outcome. Here we showed that the overexpression of S100A8/A9 in macrophages leads to increased ROS and IL-10 production by these cells, which may explain the beneficial clinical effect of these S100 molecules on kidney allograft survival. Finally, the genome-wide association study shows that individual gene polymorphisms confer only a small risk on acute rejection after kidney transplantation, and the outcome suggests that future efforts require large international collaborative studies.