Biomarkers of Lung Injury in Cardiothoracic Surgery

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CHAPTER 6

Surfactant protein D polymorphism is associated with primary graft dysfunction and survival after lung transplantation

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Submitted

Abstract

Background: Surfactant protein D is part of the innate immune system and belongs to the collectin family. Single nucleotide polymorphisms in the surfactant protein D gene are known to influence structure, function or plasma concentrations of the protein. We hypothesized that lung transplant recipients with different SP-D genotypes might have different risks for primary graft dysfunction and/or mortality.

Methods: Genotyping of the single nucleotide polymorphisms Met11Thr (T/C, rs721917), Ala160Thr (A/G, rs2243639) and Ser270Thr (T/A, rs3088308) was performed on DNA obtained from lung transplant donors and recipients from our center. Surfactant protein D genotypic variants were analyzed for their association with primary graft dysfunction and patient survival.

Results: Recipients carrying the homozygous Ala/Ala genotype of Ala160Thr were associated with increased occurrence of grade 3 primary graft dysfunction (OR: 2.03, 95% CI: 1.05 – 3.92, $p = 0.036$), and with increased mortality (HR: 1.56, 95% CI: 1.04 – 2.42, $p = 0.032$). Single nucleotide polymorphisms Met11Thr and Ser270Thr in the recipient and all three SP-D polymorphisms in the donor were not associated with PGD or mortality rate.

Conclusions: Lung transplant recipients that carry the Ala/Ala genotype of Ala160-Thr are associated with the development of grade 3 primary graft dysfunction and reduced survival.

Introduction

Lung transplantation is still the only viable option for treatment of end-stage pulmonary diseases. The median 5 year survival of recipients is approximately 55% [1], which is low as compared to other solid organ transplantations. For instance, kidney transplantation has a median 5 year survival of > 90% [2]. Of these lung transplants, 10 to 30% is affected by primary graft dysfunction (PGD), which is one of the main reasons for early and late post-transplant morbidity and mortality [3].

The hydrophilic surfactant protein D (SP-D) is involved in the innate host-defense system by regulating cytokine production from macrophages and neutrophils, and by providing direct or indirect regulation of lymphocyte activity [4, 5]. SP-D belongs to the collectin family and is produced by type II alveolar epithelial cells. Importantly, production of the protein is not exclusive to the respiratory system, as it is also produced in exocrine glands (e.g. salivary and adrenal gland) and epithelial cells (e.g. trachea, intestine, kidney) [6, 7]. The protein is assembled as a trimeric structure with the carbohydrate recognition domain connected to a collagenous domain. The carbohydrate recognition domain has high affinity for clustered oligosaccharides commonly found on the surface of viruses, bacteria, yeast, and fungi, which can lead to agglutination, phagocytosis, and removal by macrophages and neutrophils. Additionally, it has been shown that SP-D can directly inhibit growth of bacteria and fungi by increasing their membrane permeability [4, 5].

There are three known single nucleotide polymorphisms (SNPs) within the SP-D gene (SFTPD) that result in an alteration of the amino acid sequence of the protein, Met11Thr (T/C, rs721917), Ala160Thr (A/G, rs2243639) and Ser270Thr
(T/A, rs3088308), with minor allele frequencies of more than 5 percent [8, 9, 10]. The Met11Thr polymorphism is known to influence structure, function and plasma concentration of the protein. In particular the Thr/Thr genotype was significantly associated with lower SP-D serum concentrations and with lower amounts of the oligomerized form of SP-D and consequently with lower binding to bacterial ligands [11, 12]. More recently, the Ala160Thr and Ser270Thr polymorphisms have also been associated with altered SP-D plasma concentrations in infants [13] and adults [14].

Besides altering the structure, function or concentration of surfactant protein D, these single nucleotide polymorphisms have been associated with tuberculosis [15], respiratory syncytial virus infection [9] , type II diabetes [16], chronic obstructive pulmonary disease [17] and respiratory distress and/or the need for respiratory support in premature infants [13]. Recently, Aramini *et al.* showed that the Thr/Thr genotype of the Met11Thr polymorphism in donor lung allografts was associated with increased development of chronic lung allograft disease [18]. However, whether there is a role for

recipient SP-D single nucleotide polymorphisms in the development of PGD or survival after lung transplantation has not yet been elucidated. We hypothesized that this might well be the case as SP-D acts also extrapulmonary and is produced at multiple locations throughout the body having an effect on innate immunity. Therefore, we investigated the relationship of three frequent SP-D single nucleotide polymorphisms, Met11Thr, Ala160Thr or Ser270Thr, with primary graft dysfunction and mortality in the Groningen lung transplant cohort.

Materials and Methods

Study design and patients

A retrospective study included all adult lung transplant recipients (n=417) between November 1990 and May 2011 at the University Medical Center Groningen, the Netherlands. Follow-up was recorded until September 2012, resulting in a minimal follow-up period of 16 months. From this group, 33 recipients were excluded because of simultaneous transplantation of other organs (heart or liver) or retransplantation. For the analysis on the occurrence of PGD, another 33 recipients were excluded because their PGD status could not be scored, mostly because of missing x-ray images. This resulted in 351 patients available for further analysis. Due to either unavailability of DNA, failure of DNA extraction or failure of genotyping, no genotype could be assessed for 38, 72 or 35 recipients for Met11Thr, Ala160Thr and Ser270Thr, respectively. For the same reasons the genotype could not be assessed for 10, 51 or 9 donors for Met11Thr, Ala160Thr and Ser270Thr, respectively. Informed consent was given by all patients and transplant characteristics were obtained and documented.

DNA isolation and genotyping

DNA was extracted from recipient blood samples using QiAamp-colomns (QiAamp Blood Kit, Qiagen, Westburg BV, Leusden, the Netherlands) a commercial kit following the manufacturer's instructions. Genotyping of the selected SP-D single nucleotide polymorphisms (rs72191, rs2243639 and rs3088308) was performed using the Illumina VeraCode GoldenGate Assay kit (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. Genotype clustering and calling were performed using Genome Studio Software (Illumina).

Study end-points

The first end point for this study was primary graft dysfunction, occurring during the first 72 hours post transplantation, based on degree of hypoxia and x-ray infiltrates and classified according to the definition of the International Society for Heart and Lung Transplantation [19]. PGD was scored at 0, 24, 48 and 72 hours post transplantation. For the purpose of analysis, PGD was defined as any episode of grade 3 PGD developing within 72 hours post transplantation and is noted as PGD henceforth 20. The second endpoint was overall patient survival.

Statistical analysis

All values were summarized as mean and standard deviation or numbers and percentages. To compare demographic data between genotypic groups, one-way ANOVA tests were used for continuous variables and contingency tables and χ^2-tests were used for categorical variables. Differences in the allelic distribution from those expected by the Hardy-Weinberg equilibrium and the observed frequencies of the single nucleotide polymorphisms were assessed by χ^2-tests.

First, univariate analyses were performed to assess the association of donor or recipient genotypes with PGD and patient survival. χ^2-tests were performed to assess the difference in PGD between genotypic groups and Kaplan–Meier survival curves and Breslow tests were performed to assess the difference in survival between genotypic groups.

Second, multivariable logistic- and Cox regression analyses were applied to estimate the odds ratio for PGD and the hazard ratio for mortality associated with SP-D single nucleotide polymorphisms, while adjusting for age, gender, body mass index, underlying lung disease, year of transplantation, LTx type and time of surgery. The year of transplantation was included to take the possible confounding effect of changes in treatment into account.

All tests performed in order to test the (null-) hypothesis of no difference were two-sided. A probability value less than 0.05 was considered statistically significant. Statistical analyses were performed with SPSS version 18.0 (SPSS Inc., Chicago, Ill, United States).

Results

Overall, 351 patients were included for analysis, with an incidence of PGD of 30.5% (107 of 351). Recipient demographic and transplant characteristics did not significantly

Table 6.1: *Lung transplant recipient characteristics divided according to recipient genotype*

Variable	Met11Thr				Ala160Thr				Ser270Thr			
	Met/Met	Met/Thr	Thr/Thr	*p* value[a]	Ala/Ala	Ala/Thr	Thr/Thr	*p* value[a]	Ser/Ser	Ser/Thr	Thr/Thr	*p* value[a]
Patients [No.]	110	145	58		84	145	50		266	49	1	
Age [years]	45±12	48±11	47±12	0.109	47±12	48±12	44±12	0.154	46±12	48±12	63	0.325
Height [cm]	172±9	172±10	172±10	0.982	171±10	172±10	172±9	0.806	172±10	173±10	160	0.430
Weight [kg]	65±14	67±13	68±14	0.197	67±13	67±14	65±12	0.587	67±13	67±14	57	0.772
BMI [kg/m^2]	21.8±4.4	22.9±4.6	22.9±4.2	0.163	23.0±3.8	22.7±5.2	21.8±3.6	0.336	22.6±4.6	22.3±3.7	22.3	0.946
Male [No.]	47 (43%)	75 (52%)	30 (52%)	0.293	43 (51%)	68 (47%)	25 (50%)	0.764	133 (50%)	22 (46%)	0	0.534
Diagnosis [No.]				0.634				0.851				0.979
COPD	50	80	28		38	74	27		132	26	1	
Cystic fibrosis	20	26	12		16	25	11		47	11	0	
Pulmonary fibrosis	20	20	11		17	25	6		44	8	0	
Pulmonary hypertension	11	6	3		6	8	5		18	2	0	
Bronchiectasis	4	4	2		1	4	1		10	1	0	
Other	5	9	2		6	9	0		15	1	0	
Bilateral [No.]	87 (79%)	113 (78%)	47 (81%)	0.886	58 (69%)	118 (81%)	43 (86%)	0.033	213 (80%)	36 (73%)	0	0.090
Use of HLM [No.]	57 (52%)	63 (43%)	30 (52%)	0.311	41 (49%)	67 (46%)	27 (54%)	0.626	134 (50%)	18 (37%)	0	0.162
Surgery time [min]	356±138	385±124	358±151	0.990	352±146	363±128	355±134	0.817	360±133	338±131	206	0.302
CPB time [min]	116±134	97±130	123±145	0.344	106±133	107±134	116±129	0.908	111±133	85±127	0	0.332

Data are presented as mean ± standard deviation unless stated otherwise. BMI, Body mass index; COPD, Chronic Obstructive Pulmonary Disease; CPB, Cardiopulmonary Bypass; HLM, Heart-Lung machine; [a] ANOVA or Pearson χ^2 test used as appropriate.

Table 6.2: *Genotype and allele frequencies of SP-D single nucleotide polymorphisms Met11Thr, Ala160Thr and Ser270Thr among LTx recipients and from the 1000 Genomes library [20]*

						LTx recipients		1000 Genomes library (Eur)	
SNP	dbSNP	Allele	Amino acid in mature protein	Codon position from transcription start site	Genotype	No.(%)	Allele frequency (%)	(%)	Allele frequency (%)
Met11Thr	rs721917	T/C	11	31	Met/Met	110 (35.1)	58.3	35.8	58.0
					Met/Thr	145 (46.3)		44.3	
					Thr/Thr	58 (18.5)	41.7	19.9	42.0
Ala160Thr	rs2243639	A/G	160	180	Ala/Ala	84 (30.1)	56.1	39.6	61.1
					Ala/Thr	145 (52.0)		43.1	
					Thr/Thr	80 (17.9)	43.9	17.3	38.9
Ser270Thr	rs3088308	T/A	270	290	Ser/Ser	266 (84.2)	91.9	86.7	92.9
					Ser/Thr	49 (15.5)		12.5	
					Thr/Thr	1 (0.3)	8.1	0.8	7.1

differ per genotypic group, except for the number of bilateral procedures which was slightly lower in the group of patients carrying the Ala/Ala genotype of the Ala160Thr polymorphism (Table 6.1). The median follow-up time for the whole cohort was 3421 days (IQR: 1811-5640). At the end of follow-up 177 patients had died, of which 24 patients died within the first 30 days. The other deceased patients had a median survival of 1305 days (IQR: 469-2737). The patients still alive at the end of follow-up had a median follow-up of 2265 days (IQR: 464-7492).

All three single nucleotide polymorphisms were in Hardy-Weinberg equilibrium, and the observed frequencies of the alleles and genotypes were comparable to the frequencies reported in the European population in the 1000 Genomes Project (Table 6.2) [20].

One of the polymorphisms was associated with PGD. The risk for PGD was twice as large for recipients carrying the homozygous Ala/Ala genotype of Ala160Thr as compared to the dominant Ala/Thr reference group (OR: 2.03, 95% CI: 1.05 – 3.92, $p = 0.036$, Table 6.4). The Ser/Thr genotype of Ser270Thr showed a 56% reduction in risk for PGD in the crude model (OR: 0.44, 95% CI: 0.20 – 0.94, $p = 0.035$), however, after adjusting for covariates the association was no longer present (OR: 0.44, 95% CI: 0.19 – 1.03, $p = 0.058$). Donor polymorphisms were not associated with PGD (Table 6.3).

The same polymorphism that was associated with PGD was also associated with patient survival, as carriers of the homozygous Ala/Ala genotype of Ala160Thr had a 59% increased mortality rate as compared to the dominant Ala/Thr reference group (HR: 1.56, 95% CI: 1.04 – 2.42, $p = 0.032$, Table 6.4). Point estimates of the mortality rate for the crude and adjusted model were very similar. Multivariable adjusted predicted survival curves according to recipient genotype are shown in Figure 6.1. Recipient polymorphisms Met11Thr and Ser270Thr and all three donor polymorphisms were not associated with mortality rate (Table 6.3 and 6.4).

Discussion

In this study we investigated the relationship of three frequent SP-D single nucleotide polymorphisms with primary graft dysfunction and patient survival after lung transplantation. One of the SP-D polymorphisms, the Ala/Ala genotype of the Ala160Thr polymorphism, was associated with PGD and survival: patients carrying this SP-D genotype were twice as likely to develop PGD after transplantation and had a 59% increased risk to die during follow-up as compared to the dominant Ala/Thr genotype. Multivariable analysis confirmed the point estimates of the unadjusted analysis and showed that there was no confounding by the variables adjusted for. Considering that the same polymorphism is associated with PGD as well as with patient survival strengthens the probability

Table 6.3: *Multivariate logistic- and Cox regression analysis for the risk of PGD and patient survival according to donor genotype*

Donor genotype	Patients [No.]	Model	PGD in first 72h OR (95% CI)	*p* value[a]	Patient survival HR (95% CI)	*p* value[a]
Met11Thr						
Met/Met	111	Crude	0.90 (0.44-1.51)	0.687	1.15 (0.79-1.68)	0.463
		Adjusted	0.87 (0.49-1.54)	0.637	1.18 (0.81-1.74)	0.388
Met/Thr	167	Crude	1.00		1.00	
		Adjusted	1.00		1.00	
Thr/Thr	63	Crude	0.69 (0.36-1.33)	0.271	1.39 (0.90-2.14)	0.139
		Adjusted	0.74 (0.37-1.50)	0.406	1.30 (0.83-2.04)	0.252
Ala160Thr						
Ala/Ala	100	Crude	0.72 (0.40-1.29)	0.273	0.97 (0.63-1.49)	0.899
		Adjusted	0.68 (0.36-1.29)	0.235	0.88 (0.57-1.36)	0.550
Ala/Thr	133	Crude	1.00		1.00	
		Adjusted	1.00		1.00	
Thr/Thr	67	Crude	0.80 (0.42-1.53)	0.493	1.01 (0.63-1.60)	0.982
		Adjusted	0.57 (0.27-1.20)	0.139	0.81 (0.49-1.32)	0.395
Ser270Thr						
Ser/Ser	296	Crude	1.00		1.00	
		Adjusted	1.00		1.00	
Ser/Thr	45	Crude	1.02 (0.52-2.00)	0.960	1.09 (0.69-1.74)	0.706
		Adjusted	1.17 (0.56-2.43)	0.673	0.99 (0.62-1.59)	0.970
Thr/Thr	1	Crude	NA		NA	
		Adjusted	NA		NA	

[a]Logistic regression model and [b]Cox regression model with or without (Crude) corrections for age, gender, body mass index, diagnosis, year of transplantation, LTx type and time of surgery. PGD, Primary graft dysfunction; OR, Odds ratio; HR, Hazard ratio; CI, Confidence interval.

of a causal relationship, as it is also known that grade 3 PGD is strongly associated with long term mortality [21]. The SP-D polymorphisms Met11Thr and Ser270Thr in the recipient and all three SP-D polymorphisms in the donor were not associated with PGD or mortality rate.

The explanation for the effect of Ala/Ala genotype of the Ala160Thr polymorphism in the recipient on PGD and mortality after lung transplantation is not immediately evident. We speculate that recipient SP-D, produced extrapulmonary, still exerts an important effect on innate immune defense after lung transplantation. Lung allografts are constantly in contact with the outer world, which results in exposure to a vast array of pathogens, chemicals, gasses, and particles. To maintain normal lung function and defense against infection it is critical to have a good functioning innate immune system. This is evident as innate immunity plays an important role in the different complications that can follow lung transplantation, such as PGD [22], infection [23], bronchiolitis

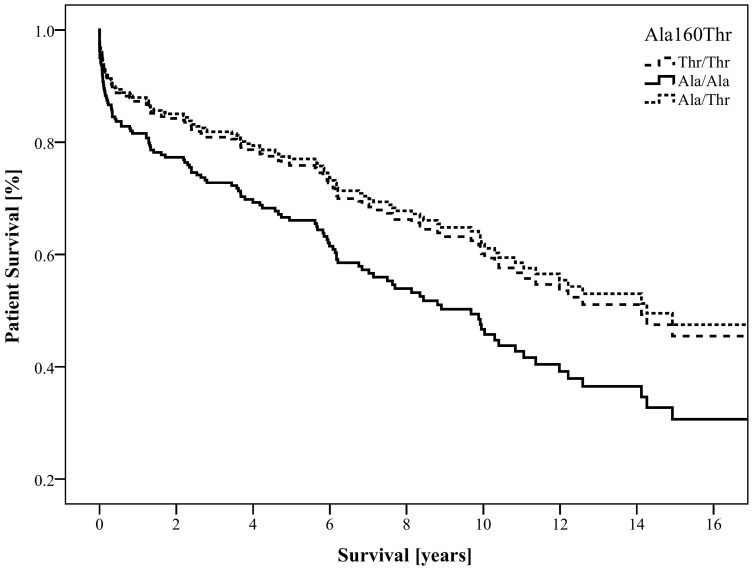
Table 6.4: *Multivariate logistic- and Cox regression analysis for the risk of PGD and patient survival according to recipient genotype*

Recipient genotype	Patients [No.]	Model	PGD in first 72h OR (95% CI)	*p* value[a]	Patient survival HR (95% CI)	*p* value[a]
Met11Thr						
Met/Met	110	Crude	1.10 (0.64-1.88)	0.738	0.84 (0.58-1.22)	0.365
		Adjusted	0.97 (0.54-1.75)	0.923	0.87 (0.59-1.28)	0.475
Met/Thr	145	Crude	1.00		1.00	
		Adjusted	1.00		1.00	
Thr/Thr	58	Crude	1.33 (0.70-2.54)	0.381	1.10 (0.72-1.70)	0.660
		Adjusted	1.28 (0.64-2.59)	0.484	1.07 (0.69-1.67)	0.752
Ala160Thr						
Ala/Ala	84	Crude	1.87 (1.05-3.33)	0.033	1.57 (1.05-2.35)	0.028
		Adjusted	2.03 (1.05-3.92)	0.036	1.59 (1.04-2.42)	0.032
Ala/Thr	145	Crude	1.00		1.00	
		Adjusted	1.00		1.00	
Thr/Thr	50	Crude	1.36 (0.67-2.75)	0.390	1.10 (0.68-1.80)	0.699
		Adjusted	1.76 (0.80-3.88)	0.159	1.06 (0.64-1.75)	0.822
Ser270Thr						
Ser/Ser	266	Crude	1.00		1.00	
		Adjusted	1.00		1.00	
Ser/Thr	49	Crude	0.44 (0.20-0.94)	0.035	1.11 (0.71-1.73)	0.661
		Adjusted	0.44 (0.19-1.03)	0.058	1.14 (0.73-1.80)	0.568
Thr/Thr	1	Crude	NA		NA	
		Adjusted	NA		NA	

[a]Logistic regression model and [b]Cox regression model with or without (Crude) corrections for age, gender, body mass index, diagnosis, year of transplantation, LTx type and time of surgery. PGD, Primary graft dysfunction; OR, Odds ratio; HR, Hazard ratio; CI, Confidence interval.

obliterans syndrome [24, 25, 26], acute rejection [27] and survival [26]. Especially the importance of innate immunity in the development of PGD has been given extensive attention in the past years [3]. For instance, toll-like receptor pathway genes were found upregulated in bronchoalveolar lavage fluid of patients with grade 3 PGD [28] and pentraxin 3 (an innate immune mediator) gene variations were associated with increased pentraxin 3 plasma concentrations and with PGD [29]. In a prior publication, the same authors showed that elevated pentraxin 3 plasma concentrations were also associated with increased risk of PGD [30].

As mentioned previously, surfactant protein D is a protein that is predominantly involved in the innate host-defense system. If we search for a mechanism by which the Ala/Ala genotype exerts its influence, one could reason that the function of the protein is altered; hereby compromising the innate immune system of the recipient. The Ala160Thr polymorphism lies on the collagen like domain of SP-D and since the polymorphism is



Patients still at risk

Ala160Thr	Year:	0	2	4	6	8	10	12	14	16
Ala/Ala		92	61	42	31	20	13	9	4	3
Ala/Thr		148	119	85	62	44	33	15	8	4
Thr/Thr		52	40	33	27	21	15	13	10	4

Figure 6.1: *Multivariable adjusted survival curves for recipients grouped according to their genotype for Ala160Thr. Survival estimates were calculated by means of Cox regression analysis and were adjusted for age, gender, body mass index, underlying lung disease, year of transplantation, LTx type and time of surgery. The Ala/Ala genotypic group has a 59% increased mortality rate as compared to the Ala/Thr group (p = 0.032, Table 6.4).*

changing one of the amino acids in the protein, it is possible that this results in altered plasma concentration, protein oligomerization or protein function. While an association between genotype and binding to bacterial ligands has been reported for the Met11Thr polymorphism [12], the association for Ala160Thr polymorphism has been limited to SP-D plasma concentrations thus far [13]. In the latter study the Ala/Ala genotype was associated with lower SP-D plasma concentrations. However, since we did not formally measure SP-D plasma concentrations or establish protein oligomerization, limited conclusions can be drawn on the mechanistic link between the Ala160Thr polymorphism, PGD and/or patient survival. Furthermore, we recognize that another single nucleotide polymorphism in linkage with this polymorphism could be responsible for the observed

associations.

Although our study describes one of the larger cohorts evaluating genetic predisposition in lung transplant recipients, it should be replicated in a cohort of similar size to confirm its findings. The importance of replication is illustrated by a study of Foreman *et al.*, were associations between SP-D genotypes and chronic obstructive pulmonary disease could not be replicated among four different study cohorts [17].

In conclusion, we showed that genetic variation in the SP-D gene in the recipient was linked with increased risk for PGD and increased hazard for mortality, where recipients carrying the Ala/Ala genotype of the Ala160Thr polymorphism were predisposed to a worsened outcome. We have once more shown that transplantation outcome is not only dependent on environmental factors and/or transplantation characteristics, but also on the genetic predisposition of the recipient. Future studies should prospectively investigate the mechanism by which Ala/Ala carriers are predisposed to reduced survival. These new insights could, in turn, lead to an improved treatment strategy.

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