

# A Promoter Polymorphism of the Alpha 8 Integrin Gene and the Progression of Autosomal-Dominant Polycystic Kidney Disease

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## Key Words

Alpha 8 integrin · Promoter · Autosomal-dominant polycystic kidney disease · Association study · End-stage renal disease

## Abstract

**Background/Aims:** Dysregulation of integrins is a feature of tissue remodeling in autosomal-dominant polycystic kidney disease (ADPKD). The alpha 8 beta 1 integrin ( $\alpha 8\beta 1$ ) affects kidney development and the susceptibility to renal injury in mice. We investigated whether the -414 T/C polymorphism in the promoter region of the alpha 8 integrin chain gene (ITGA8) is associated with the progression of renal disease in ADPKD. **Methods:** Genotyping for the -414 T/C polymorphism was performed by allelic separation using RT-PCR in 294 patients with ADPKD. Alpha 8 integrin expression was detected by RT-PCR and immunohistochemistry. **Results:** 41% of the study population reached end stage renal disease at a mean age of  $51 \pm 12$  years. The frequency of the -414 C allele was 0.194 in ADPKD. C allele carriers (CC and TC genotypes) were compared with patients homozygous for the T allele (TT genotype). Kaplan-Meier analysis revealed that end-stage renal failure occurred at a significantly younger age in TT homozygotes (median age, 47 years; 95% CI, 46–49 years) than in C allele carriers (median age, 51 years; 95% CI, 49–53 years;  $p = 0.046$  by the log-rank test). When parameters of ADPKD patients were compared between genotype by analysis of variance, only age at onset of end-stage renal

failure was significantly different ( $p = 0.026$ ) whereas age at onset of hypertension, body surface area, 24-hour systolic and diastolic blood pressure did not differ. In kidneys of ADPKD, expression of alpha 8 integrin is increased and found de novo in cystic epithelia. **Conclusion:** A polymorphism of the ITGA8 promoter modifies the progression of renal failure in ADPKD.

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## Introduction

The alpha 8 beta 1 integrin ( $\alpha 8\beta 1$ ) belongs to a family of heterodimeric cell-matrix receptors consisting of one  $\alpha$  and one  $\beta$  subunit [1, 2]. Altered expression of  $\beta 1$  integrins as well as their matrix ligands has been described in chronic kidney diseases, including several forms of glomerulonephritis [3, 4], diabetic nephropathy [5] or acute renal failure [6]. Moreover, upregulation of  $\beta 1$  integrins was shown in animal models of glomerular disease [7, 8]. The role of integrins for the severity or progression of glomerular disease is still a matter of investigation and in some cases controversial:  $\alpha 1\beta 1$  integrin apparently exerted profibrotic effects in a model of glomerulonephritis in the rat [9], whereas lack of  $\alpha 1\beta 1$  led to severe glomerulosclerosis following glomerular injury induced by adriamycin [10]. The integrin chain  $\alpha 8$  is expressed in mesenchymal and neuronal cells and heterodimerizes exclusively with the  $\beta 1$  chain [11, 12]. Integrin  $\alpha 8\beta 1$  is a

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receptor for fibronectin, vitronectin, tenascin C, osteopontin and nephronectin [13–15]. In the healthy kidney, expression of the  $\alpha 8$  chain is restricted to glomerular mesangial cells and vascular smooth muscle cells [16]. An increase in  $\alpha 8$  integrin expression was detected in models of glomerulonephritis [16] and glomerular hypertension [17]. In renal interstitial fibrosis, some activated myofibroblasts express  $\alpha 8$  integrin de novo [17]. Studies of the sequelae of glomerular hypertension in these mice revealed a role for integrin  $\alpha 8\beta 1$  in maintaining the integrity of the glomerular capillary tuft during mechanical stress [17]. Lack of  $\alpha 8\beta 1$  leads to abnormalities of kidney development and to an increased susceptibility to renal injury in mice. Mice with a homozygous deletion of the  $\alpha 8$  integrin chain have a reduced renal mass due to impaired epithelial-mesenchymal interactions in early stages of kidney development [18]. However, glomeruli and vessels are apparently normal with only subtle morphological alterations and no overt functional abnormalities [19].

Autosomal-dominant polycystic kidney disease (ADPKD) is the most common hereditary renal disease, and frequently leads to end-stage renal disease (ESRD) [20]. In polycystic kidney diseases (including ADPKD), a dysregulation of several integrin chains was detected in tubular and cystic epithelial cells [21]. Colocalization of integrin  $\alpha 2\beta 1$  and polycystin-1, the protein most frequently affected in ADPKD, was described in focal adhesion complexes [20]. Moreover, it was shown that suppression of polycystin-1 was associated with an increase of  $\alpha 2\beta 1$  integrin and subsequently increased cell survival [22]. Accumulation of tenascin, a ligand for  $\alpha 8\beta 1$  integrin, was described in ADPKD [20]. Whether or not  $\alpha 8\beta 1$  integrin is involved in the pathogenesis and/or progression of ADPKD is completely unknown. However,  $\alpha 8$  integrin-deficient mice display increased expression of  $\alpha 2\beta 1$  integrin [19]. Moreover, both polycystin and  $\alpha 8\beta 1$  integrin seem to play a role for sprouting of the ureteric bud during early kidney development [20]. We tested whether a polymorphism in the promoter region of the integrin  $\alpha 8$  chain gene (ITGA8) is associated with the progression of renal failure in ADPKD.

## Materials and Methods

### Patients

A cohort of 294 patients with autosomal polycystic kidney disease (ADPKD) from southern Germany was included in our study. Eighty percent of these patients were hypertensive. Data on the age at onset of ESRD, gender, body mass index and hyperten-

sion status were collected from all patients. Data on 24-hour ambulatory systolic and diastolic pressure, and the age at diagnosis of hypertension was available for the majority of patients. The study protocol was approved by the local Ethics Committee (University of Erlangen-Nuremberg, Germany). All patients provided informed consent.

### Immunohistochemistry

Frozen renal specimens were from kidney explants from 7 ESRD patients with ADPKD. Nephrectomies were performed because of the size of the kidneys in preparation for future kidney transplantations. Five control kidney specimens were taken from healthy parts of tumor explants. The use of anonymous pathologic material was approved by the local Ethics Committee (University of Erlangen-Nuremberg, Germany). For detection of  $\alpha 8$  integrin, 2- $\mu$ m cryosections were prepared. Staining of cryostat sections was performed as described elsewhere [23]. In brief, sections were fixed with acetone for 10 min. Unspecific staining was reduced by blocking with fetal calf serum for 10 min, then the sections were layered with the primary anti- $\alpha 8$  integrin antibody (rabbit anti-mouse, cross-reacting with human) in a 1:50 dilution overnight at 4°C. After washing with tris-buffered saline, sections were layered with CY3-labeled anti-rabbit IgG (DAKO Diagnostica, Hamburg, Germany) as secondary antibody for 1 h in the dark (1:400). Stained sections were embedded in mowiol and evaluated in a Leitz Aristoplan microscope (Leica Instruments).

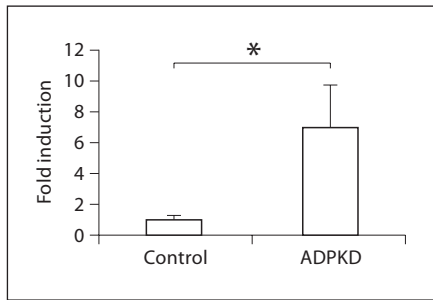
### Real-Time PCR

Renal tissue (10 mg) from frozen kidney explants from 5 ESRD patients with ADPKD and 5 control kidney specimens from healthy parts of tumor explants was homogenized in 500  $\mu$ l of RLT buffer reagent (Quiagen, Hilden, Germany) with an ultraturax for 30 s, and total RNA was extracted with RNeasy Mini columns (Quiagen) according to the standard protocol. First-strand cDNA was synthesized with TaqMan reverse transcription reagents (Applied Biosystems, Darmstadt, Germany) using random hexamers as primers. Final RNA concentration in the reaction mixture was adjusted to 0.1 ng/ $\mu$ l. Reactions without Multiscribe reverse transcriptase were used as negative controls for genomic DNA contamination. RT products were diluted 1:1 with dH<sub>2</sub>O before the PCR procedure. PCR was performed with an ABI PRISM 7000 Sequence Detector System and SYBR Green reagents (Applied Biosystems) according to the manufacturer's instructions.

For amplification of the mouse  $\alpha 8$  integrin cDNA, the forward primer was 5'-TCA AGG CGA GGA ACA GCA A-3' and the reverse primer was 5'-CCT TGG GAA CCC GAT GGT-3'. The relative amount of the specific mRNA was normalized with respect to 18S rRNA. Primers used for amplification of 18S cDNA were: forward primer 5'-TTG ATT AAG TCC CTG CCC TTT GT-3' and reverse primer 5'-CGA TCC GAG GGC CTC ACT A-3'. All samples were run in triplicate.

### Allelic Separation of the -414 T/C Polymorphism by TaqMan PCR

The human gene for the  $\alpha 8$  integrin chain (ITGA8) is localized on chromosome 10p13. PKD-1 is localized on 16p13 and PKD-2 on 4q21. Genotyping for the -414 T/C polymorphism was performed by allelic separation using RT-PCR in 294 patients with ADPKD. The following primers and probes were used: forward



**Fig. 1.** Expression of mRNA for  $\alpha 8$  integrin in ESRD kidney explants of ADPKD patients compared to control kidneys evaluated by RT-PCR.  $n = 5$  per group. Data are means  $\pm$  SEM. \*  $p < 0.05$  ADPKD vs. controls.

primer: GCGCTGGGTGCCTCTT, reverse primer: GGTGACAGAGACGAACTGATTGT, reporter 1: ATTTCTATTGCAGTCCCATC-vic and reporter 2: CTATTGCGGTCCCATC-fam.

#### Analysis of Data

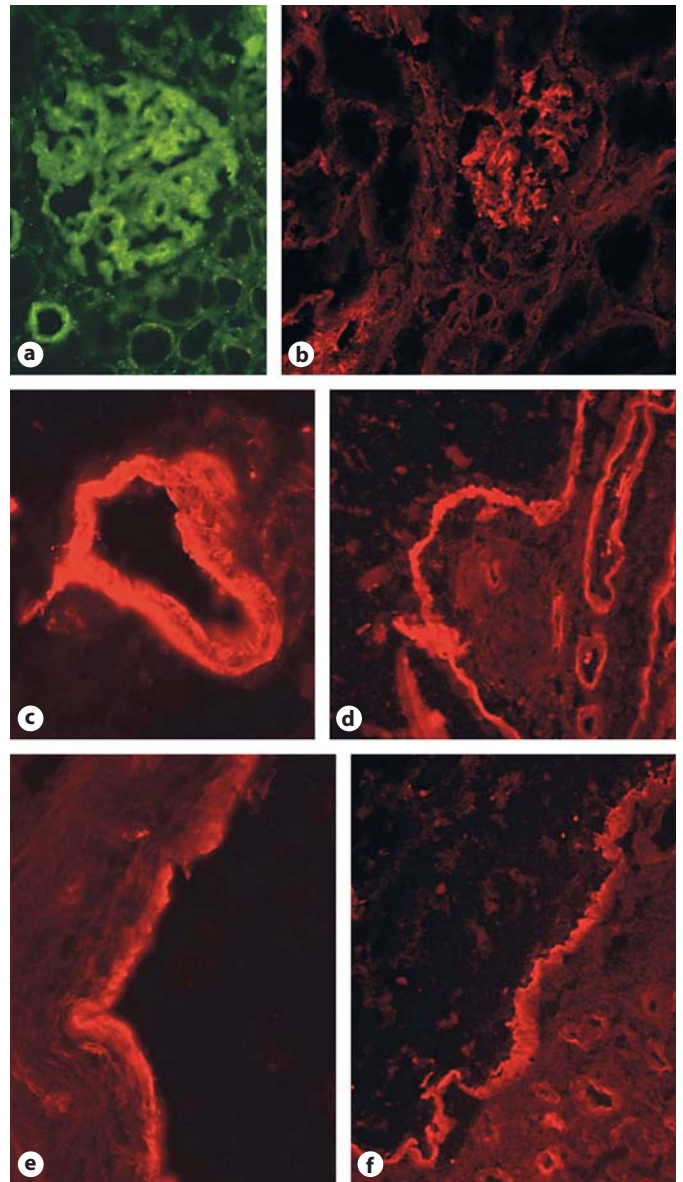
Presence of ESRD and age at onset were compared between genotypes by  $\chi^2$  and Kaplan-Meier analysis. Blood pressure levels and age of onset of ESRD were compared by the two-tailed t test.  $p < 0.05$  was considered significant. The procedures were carried out using the SPSS version 13.0 software (SPSS Inc., Chicago, Ill., USA). Values are displayed as means  $\pm$  SEM.

#### Results

An increase in  $\alpha 8$  integrin expression was detected in the tissue of kidney explants from ESRD patients with ADPKD compared to control kidneys (fig. 1). The localization of  $\alpha 8$  integrin was evaluated in sections of ADPKD explants and control kidney tissue. Similar to the findings in control renal tissue (fig. 2a),  $\alpha 8$  integrin was detected in the vasculature and in the glomerular mesangium of cystic kidneys (fig. 2b, c). However, in ADPKD kidneys de novo expression of  $\alpha 8$  integrin was detected in epithelial cells, mainly in cyst walls (fig. 2d–f).

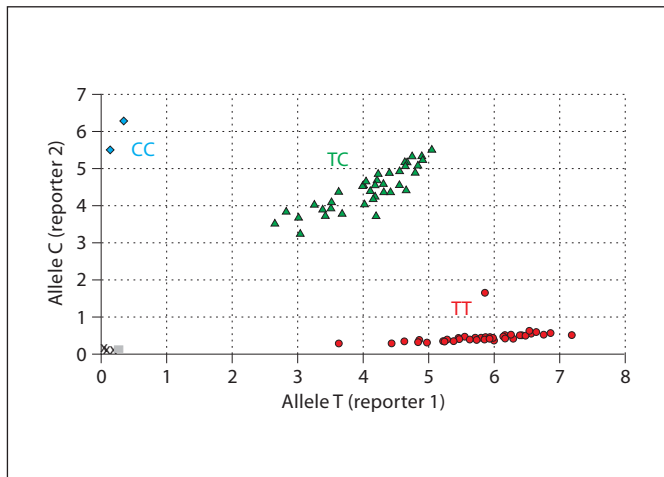
Figure 3 shows allelic separation of the CC, TC and TT genotype. 186 ADPKD patients were of the TT genotype, 102 of the TC genotype and 6 of the CC genotype. The distribution of genotypes was consistent with the Hardy-Weinberg equilibrium. The frequency of the -414 C allele was 0.194 in ADPKD patients. Due to the very low number of CC homozygotes (6 of 294), C allele carriers (CC and TC genotypes) were compared with patients homozygous for the T allele (TT genotype).

41% of the study population reached ESRD at a mean age of  $51 \pm 12$  years. The number of patients on renal

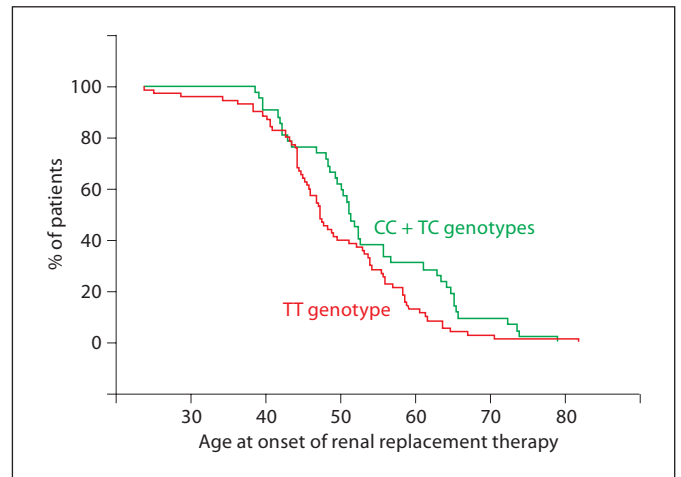


**Fig. 2.** Localization of  $\alpha 8$  integrin in ESRD kidney explants of ADPKD patients by immunofluorescent staining of renal sections. **a** Control kidney.  $\alpha 8$  integrin is localized to the mesangium of the glomerulus and to the media of the renal vasculature. **b–f** ADPKD kidney:  $\alpha 8$  integrin is localized to glomeruli (**b**), renal arterial vasculature (**c**) and cyst walls (**d–f**).

replacement therapy was not affected by the ITGA8 genotype. However, C allele carriers needed renal replacement therapy at a significantly higher age: Kaplan-Meier analysis revealed that ESRD occurred earlier in TT homozygotes (median age, 47 years; 95% CI, 46–49 years) than in C allele carriers (median age, 51 years; 95% CI,



**Fig. 3.** Allelic separation by TaqMan RT-PCR analysis. Unanimous identification of the genotypes was achieved.



**Fig. 4.** Age at the onset of renal replacement therapy of the TT and CC/TC genotypes. Kaplan-Meier analysis reveals a significantly earlier onset of ESRD in individuals carrying the TT genotype.

**Table 1.** Data from all ADPKD patients

	TT	CC + TC	Test	p value
Male:female	100:86	54:54	$\chi^2$	0.547
Smoker:nonsmoker	38:111	20:65	$\chi^2$	0.756
ESRD yes:no	78:108	44:64	$\chi^2$	0.902
Age at onset of ESRD, years	49 ± 10	54 ± 11	t test	0.026
Age at hypertension diagnosis, years	34 ± 14	36 ± 13	t test	0.171
24-hour ambulatory systolic BP, mm Hg	137 ± 15	137 ± 16	t test	0.838
24-hour ambulatory diastolic BP, mm Hg	85 ± 10	86 ± 10	t test	0.595

**Table 2.** Data from ADPKD patients on renal replacement therapy

	TT	CC + TC	Test	p value
Male:female	48:30	28:16	$\chi^2$	0.488
Smoker:nonsmoker	9:41	5:21	$\chi^2$	0.563
Age at onset of ESRD, years	49 ± 10	54 ± 11	t test	0.026
Age at hypertension diagnosis, years	32 ± 18	35 ± 13	t test	0.349
24-hour ambulatory systolic BP, mm Hg	139 ± 17	141 ± 19	t test	0.620
24-hour ambulatory diastolic BP, mm Hg	84 ± 11	85 ± 10	t test	0.631

49–53 years;  $p = 0.0463$  by the log-rank test) as illustrated in figure 4. When parameters of all ADPKD patients were compared between genotype by analysis of variance, only age at onset of ESRD was significantly different ( $p = 0.026$ ) whereas age at onset of hypertension, body weight, height, 24-hour systolic and diastolic blood pressure did not differ (table 1). The blood pressure data are of limited

value, as they were obtained at different stages of renal failure and under different medications. A comparison of the collected data only from ADPKD patients on renal replacement therapy revealed no differences in the age at onset of hypertension, 24-hour systolic and diastolic blood pressure between the genotypes (table 2).

## Discussion

In the present study, we demonstrate that the -414 T/C polymorphism of the  $\alpha 8$  integrin chain gene promoter is associated with the age at onset of ESRD in ADPKD. This promoter polymorphism is present in the European population in a distribution which is consistent with the Hardy-Weinberg equilibrium. As the -414 T/C polymorphism occurs at the binding site for the transcription factor common factor-1 [24], it is conceivable that binding of this transcription factor and subsequently the transcription of the  $\alpha 8$  integrin chain gene could be affected by this polymorphism. Common factor-1 was described to serve as a transcriptional activator, e.g. of the *c-myc* gene or the skeletal  $\alpha$ -actin, and tends to be ubiquitously expressed in all tissues investigated [24]. However, there is at present no experimental evidence to support the notion that this promoter polymorphism reflects differences in  $\alpha 8$  integrin expression levels. The small number of specimens from ADPKD kidneys which were available for RT-PCR analysis precluded a comparison of  $\alpha 8$  integrin mRNA expression between genotypes.

Nevertheless, we detected an increase of  $\alpha 8$  integrin mRNA expression and de novo  $\alpha 8$  integrin protein expression in the walls of cysts from ADPKD kidney sections. Changes in  $\alpha 8$  integrin expression have not been described in human kidney diseases before, although there is some evidence that expression of  $\alpha 8$  integrin is altered in a rat model of hypertensive nephropathy [17] where it seems to contribute to maintaining the structural integrity of glomeruli. A dysregulation of other integrins was found in ADPKD. An upregulation of the collagen receptor component  $\alpha 1$  integrin chain was detected in normal and cystic collecting duct cells of ADPKD [21]. The distribution of integrin subunits  $\alpha 2$ ,  $\alpha 3$  and  $\alpha 6$  was irregular in cyst walls [21]. Joly et al. [25] demonstrated increased integrin  $\beta 4$  gene expression associated with increased expression of its ligand laminin-5 in ADPKD. Thus, it is conceivable that changes in integrin expression influence the development of renal cysts, possibly via altering cell-matrix interactions [20]. Upon activation, integrins colocalize with a number of focal adhesion proteins to form focal adhesions, which link cytoskeletal components in the cell to extracellular matrix outside the cell [20, 26]. Polycystin-1, the protein most commonly affected in ADPKD, seemed to be localized to these focal adhesion complexes [20]. Moreover, another molecule present in focal adhesions, tensin, was shown to be of critical importance for preventing cyst formation [27]. Mice deficient for tensin develop renal cystic disease resembling human ADPKD.

Another feature of ADPKD is a change in the composition of matrix molecules, sometimes associated with interstitial fibrosis [21, 25, 28]. Particularly, a marked increase in tenascin deposition frequently accompanies ADPKD [28, 29]. As tenascin is a ligand for  $\alpha 8$  integrin [13] and as it is expressed in the subepithelial lining of cyst walls [28], which in turn are shown here to express  $\alpha 8$  integrin, signaling events from the matrix via  $\alpha 8$  integrin might influence cellular functions of cystic epithelial cells. A typical feature of these cells is abnormal cell proliferation [30], which can also be detected in animal models of polycystic kidney disease [31]. A contribution of  $\alpha 8$  integrin to the regulation of cell proliferation was described in mesangial cells [32]. Expression of  $\alpha 8$  integrin was associated with a decrease of cell proliferation. Thus, the de novo expression of  $\alpha 8$  integrin in cystic epithelia might be considered as an attempt to reduce pathologic cell growth in ADPKD. On the other hand, expression of  $\alpha 8$  integrin in vascular smooth muscle cells proved to be important for the maintenance of the mesenchymal phenotype of the cells [33]. Expression of  $\alpha 8$  integrin might indicate that epithelialization of cyst walls is reduced, a notion which is supported by the finding that in the cyst epithelia of ADPKD kidneys alterations in cell polarity were found [34]. Membrane polarization is essential for the maturation of renal epithelia. A mispolarization [35] or underexpression [36] of E-cadherin detected in cyst walls could also be a characteristic of a mild dedifferentiation in cyst epithelia.

Alternatively,  $\alpha 8$  integrin might be involved in the development of fibrotic changes in ADPKD, as suggested by Levine et al. [37] and Bouzeghrane et al. [38] for pulmonary, hepatic and myocardial fibrosis, respectively. We cannot rule out this possibility but consider it unlikely for two reasons. First, in our ADPKD kidney sections,  $\alpha 8$  integrin immunoreactivity was primarily localized in cyst walls, not in the areas of fibrosis which were abundantly present in these end-stage kidneys. Second, our previous experiments in  $\alpha 8$  integrin-deficient mice [17] did not support the notion that this integrin promotes fibrosis in the kidney. The lack of this integrin aggravated the hypertension-induced glomerular damage [17]. An increased susceptibility to hypertensive kidney injury could also contribute to the effect of the  $\alpha 8$  integrin gene promoter polymorphism on the course of ADPKD. Association studies have investigated the potential role of genes involved in the regulation of blood pressure and hypertensive target organ damage, including endothelial nitric oxide synthase [39, 40] and angiotensin-converting enzyme [41, 42], as modifier genes for ADPKD. These

studies, however, yielded conflicting results as to the importance of these genes for the time course of renal failure.

Several limitations of our study must be considered. First, the association design does not permit to establish a cause-and-effect relationship. Second, there are no data to prove that the -414 T/C promoter polymorphism affects the transcription of the gene, and we cannot exclude the possibility that the apparent effect of the polymorphism may be due to other polymorphisms in linkage disequilibrium with -414 T/C. Third, the number of patients with ESRD was not large for an association study. Previously published association studies in ADPKD patients [39, 40, 42], however, described cohorts of similar size. Finally, we did not genotype subjects for the *PKD1* or *PKD2* mutations, and we are therefore unable to exclude an interaction between the  $\alpha 8$  integrin promoter polymorphism and the *PKD* genotype.

Despite these limitations, our results point to the role of cell-matrix interactions for the development of cystic kidney diseases, especially in ADPKD. A dysregulation of molecules involved in the regulation of cell-matrix interactions may therefore contribute to renal tissue remodeling preceding cyst formation. Our data raise the hypothesis that the  $\alpha 8$  integrin chain plays a role in this process.

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