Univerzita Karlova v Praze

1. lékařská fakulta

Studijní program: Doktorské studium biomedicíny

Studijní obor: Molekulární a buněčná biologie, genetika a virologie

Ing. Irena Brabcová

MOLEKULÁRNÍ MARKERY OVLIVŇUJÍCÍ PŘEŽITÍ TRANSPLANTOVANÉ LEDVINY A PROGRESI GLOMERULOPATIÍ

MOLECULAR MARKERS WITH IMPACT ON KIDNEY GRAFT SURVIVAL AND GLOMERULOPATHIES PROGRESSION

Disertační práce

Školitelé:

Prof. MUDr. Ondřej Viklický, CSc.

MUDr. Jiří Lácha, CSc.

Praha, 2010

Děkuji svým školitelům za odborné vedení, nápady a cenné připomínky při zpracování této práce. Školiteli MUDr. Jiřímu Láchovi, CSc. děkuji zejména za pozitivní motivaci, podporu a pomoc při počátku řešení projektů předkládané práce. Školiteli Prof. MUDr. Ondřejovi Viklickému, CSc. děkuji za ochotu, se kterou se po náhlé smrti MUDr. Láchy ujal vedení mé práce, za cenné konzultace, nové náměty a pomoc při jejich realizaci. Zvláštní poděkování patří celému kolektivu Transplantační laboratoře, zejména Ing. Petře Hřibové, Ph.D., Mgr. Evě Girmanové, Ing. Kateřině Hyklové a Romaně Poláčkové za podporu, pomoc a vytváření příjemné pracovní atmosféry při řešení dílčích projektů mé práce. V neposlední řadě děkuji MUDr. Janu Petráškovi a Mgr. Jeleně Skibové za pomoc a rady při statistickém zpracování dat. Poděkování patří také všem spolupracujícím lékařům z Kliniky nefrologie Institutu klinické a experimentální medicíny, Všeobecné fakultní nemocnice v Praze a Institutu lékařské imunologie Humboldtovy Univerzity v Berlíně.

Děkuji své rodině a blízkým, bez jejichž pochopení a podpory by tato práce nemohla vzniknout.

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem řádně uvedla a citovala všechny použité prameny a literaturu. Současně prohlašuji, že práce nebyla využita k získání jiného nebo stejného titulu.

Souhlasím s trvalým uložením elektronické verze mé práce v databázi systému meziuniverzitního projektu Theses.cz za účelem soustavné kontroly podobnosti kvalifikačních prací.

V Praze, 25. 10. 2010

Irena Brabcová

Podpis

Identifikační záznam:

BRABCOVÁ, Irena. *Molekulární markery ovlivňující přežití transplantované ledviny a progresi glomerulopatií. [Molecular markers with impact on kidney graft survival and glomerulopathies progression]*. Praha, 2010. Počet stran 62, 4 přílohy. Disertační práce. Univerzita Karlova v Praze, 1. lékařská fakulta, Institut klinické a experimentální medicíny. Vedoucí závěrečné práce Prof. MUDr. Ondřej Viklický, CSc.

OBSAH

ABSTRAKT

Na progresi chronických glomerulopatií, i na odhojování transplantované ledviny má vliv řada prozánětlivých cytokinů, jejichž role v patogenezi poškození není dosud detailně popsána. Cílem této disertační práce bylo identifikovat spolehlivé markery rizika progrese renální dysfunkce a přispět tak k efektivnější léčbě pacienta.

Analyzovány byly vzorky biopsií nativních ledvin s histologicky potvrzenou glomerulopatií a biopsií ledvinných štěpů. Intrarenální genové exprese byly měřeny metodou RT-qPCR, jednonukleotidové polymorfizmy detekovány metodami založenými na PCR-RFLP. Pro hodnocení mononukleárního infiltrátu bylo využito imunohistochemické barvení. Na naměřených datech byla provedena statistická analýza.

V době diagnózy IgA nefropatie byla zjištěna korelace genových transkriptů *TGF-β1, HGF, BMP7, MCP-1* a *RANTES* a mononukleárního infiltrátu se sníženou renální funkcí a proteinurií. Ve dvouletém sledování závisela progrese IgAN na tíži chronické vaskulopatie a na míře exprese genu pro TGF-β1. Pacienti po transplantaci ledviny s dysfunkcí štěpu a zvýšenou intrarenální expresí genu pro TGF-β1 a MCP-1 měli zkrácenou dobu přežívání transplantované ledviny. V době biopsie měli pacienti s dysfunkcí štěpu upregulovány také mRNA genů pro IL-10, TGF-β1, IL-6, MCP-1, RANTES a TNF-α. Míra tubulitidy ve štěpu korelovala s intrarenální expresí *MCP-1*, *RANTES* a *TGF-β1*. Žádný z funkčně relevantních polymorfizmů v genech *TNF-a*, *MCP-1*, *RANTES, CCR2*, *CCR5*, *IFN-γ* a *TGF-β1* nezvyšoval riziko subklinické rejekce, akutní rejekce nebo nefropatie štěpu u sledovaných pacientů po transplantaci ledviny.

Závěrem lze konstatovat, že tato studie přispívá k identifikaci nových biomarkerů, pomocí kterých lze identifikovat nemocné v riziku progrese IgA nefropatie a komplikací po transplantaci ledviny.

Klíčová slova: IgA nefropatie, dysfunkce štěpu, genová exprese, genový polymorfizmus.

ABSTRACT

The progression of chronic glomerulopathy and graft rejection is affected by a number of proinflammatory cytokines, whose role in the pathogenesis of damage is poorly understood. The aim of this dissertation was to identify reliable risk markers of renal dysfunction progression and thereby contribute to a more effective patient treatment.

Human native kidney biopsies with histologically confirmed diagnosis of glomerulopathy or kidney graft biopsies were analysed. Intrarenal gene expressions were measured by RT-qPCR. Single nucleotide polymorphisms were detected by methods based on PCR-RFLP. Immunohistochemical staining was used to identify and quantify the mononuclear cell infiltration.

Gene expression of TGF-β1, HGF, BMP7, MCP-1, RANTES and mononuclear cell infiltration were associated with poor renal function and proteinuria at the time of IgA nephropathy diagnosis. Progression of IgA nephropathy during the 2-year follow-up was shown to be dependent on the degree of chronic vasculopathy and TGF-β1 expression in the kidney. Patients with graft dysfunction and enhanced intrarenal expression of TGF-β 1, MCP-1 had significantly shorter graft survival. Higher mRNA expression of IL-10, TGF-β 1, IL-6, MCP-1, RANTES and TNF-α was observed in patients with graft dysfunction presented at the time of biopsy. Correlation between the degree of allograft tubulitis and MCP-1, RANTES and TGF-β1 gene expression was found. None of the examined functionally relevant gene polymorphisms increased the risk of subclinical rejection, acute rejection or allograft nephropathy in selected patients.

In conclusion, this study helps to find new biomarkers of IgA nephropathy progression and complications after kidney transplantation and thereby identify patients with increased risk.

Key words: IgA nephropathy, graft dysfunction, gene expression, gene polymorphism

ÚVOD

Z lékařské praxe je známo, že onemocněním ledvin trpí přibližně 10% populace. Jde často o závažná onemocnění, která mohou vést k nezvratnému selhání ledvin. V České republice žije t. č. 10 000 nemocných trpících nezvratným selháním ledvin. Z nich žije 4 000 s funkční transplantovanou ledvinou, zbytek je léčen dialyzačními metodami [1]. Transplantace ledviny představuje metodu volby léčby nezvratného selhání ledvin, protože je spojena s delším přežitím nemocných a s lepší kvalitou jejich života.

Nejčastější primární glomerulopatií, která vede k selhání ledvin až u 20% pacientů, je IgA nefropatie (Mb. Berger, IgAN). Představuje nejčastější diagnózu v bioptických registrech také v České republice [2]. Mechanizmy, které vedou k progresi IgAN do selhání nejsou dosud detailně popsány. Podobně mechanizmy progrese dysfunkce transplantované ledviny do selhání jsou předmětem intenzivního výzkumu. Ukazuje se, že jak na progresi chronických glomerulopatií včetně IgAN, tak na odhojování ledvinného štěpu má vliv řada molekulárních mechanizmů, které nejsou dosud přesně identifikovány a jejichž role v patogenezi poškození nejsou detailně popsány. Výzkum těchto mechanizmů byl i cílem této disertační práce.

Nalezení prognostických markerů, které ovlivňují patogenezi a progresi IgAN či potransplantčních komplikací by mohlo pomoci identifikovat rizikové pacienty, individualizovat jejich léčbu nebo monitorovat účinnost terapie. Takto pojatý výzkum je v souladu s v současnosti stále více diskutovaným novým přístupem k onemocnění pacienta, tzv. „P4 medicínou“ (personalizovanou, prediktivní, preventivní, participační), která přetváří podstatu léčby od reaktivní k preventivní. Takový přístup by přinesl včasnou detekci onemocnění, umožnil by výběr optimální terapie pro každého pacienta a snížení nepříznivých účinků terapie díky včasnému efektivnějšímu hodnocení individuální reakce na léčbu. Zároveň usiluje o zdokonalení výběru nových biochemických cílů pro výzkum

léčiv a v neposlední řadě má za následek zkrácení času a snížení finančních nákladů na léčbu díky tomu, že klade důraz především na prevenci onemocnění.

IgA nefropatie (Mb. Berger, IgAN)

IgAN probíhá velmi často bez klinických příznaků a onemocnění je tudíž rozpoznáno až ve fázi selhání ledvin. Za nezávislé rizikové faktory progrese onemocnění jsou podobně jako u jiných glomerulopatií považovány hlavně hypertenze a proteinurie. Jedním z možných vysvětlení mechanizmu renálního postižení proteinurií je fakt, že filtrovaný protein je zpětně reabsorbován v buňkách proximálního tubulu a dále je v těchto buňkách degradován lysosomálním aparátem. Z experimentálních poznatků je známo, že zvýšení koncentrace albuminu, IgG nebo transferinu vede v tubulárních buňkách ke zvýšení syntézy endotelinu-1 a způsobí aktivaci transkripčního faktoru NF-kB v buněčném jádře [3]. Nukleární faktor kappa B (NF-kB) je transkripční faktor který zvyšuje expresi prozánětlivých chemokinů a cytokinů. Zvýšení koncentrace některých cytokinů v intersticiu má pak za následek indukci proliferace fibroblastů a zvýšení produkce komponent extracelulární matrix, tedy fibrotizaci. Z literatury jsou známa data ukazující, že proteinurie, hypertenze a snížení glomerulární filtrace v době biopsie jsou významnými rizikovými faktory progrese onemocnění [4-7], zatímco snížení proteinurie během léčby je důležitým faktorem zpomalujícím progresi [8]. Je známo, že u pacientů s IgAN dochází k odchylkám v tvorbě protilátek IgA v důsledku čehož je v séru, v cirkulujících imunokomplexech a v glomerulech přítomno velké množství polymerního IgA1. Snížené odstraňování IgA1 a imunokomplexů obsahujících IgA1 z cirkulace v důsledku poruchy glykosilace nebo narušení fagocytózy usnadňuje jejich ukládání v mezangiu glomerulů [9].

Problémem u tohoto onemocnění je velice obtížné usuzování na imunologickou aktivitu onemocnění, neboť postižení může přetrvávat až desítky let bez klinické manifestace. Histologický nález i klinické hodnoty jsou markery většinou již nevratného

renálního poškození a neodráží aktivitu onemocnění, jejíž znalost by mohla pomoci včas predikovat vývoj choroby.

V některých pracích je přítomnost renální dysfunkce, proteinurie a hypertenze v čase provedení diagnózy v přímém kontextu s intersticiální fibrózou a asociuje s progresí onemocnění [10]. Nicméně přesnost těchto klinických markerů není dostatečná pro spolehlivou predikci rizika progrese IgAN a vytypování rizikových pacientů, kteří potřebují úpravu léčebné terapie. V poslední době se ukazuje, že expresní analýza kandidátních genů hrajících roli v patogenezi onemocnění by mohla být vhodná pro sledování imunitní aktivity IgAN a tak přispět k individualizaci léčby.

Výběr vhodných kandidátních genů se opírá většinou o výsledky získané na experimentálních modelech. V případě IgAN je intersticiální fibróza a tubulární atrofie přímým následkem renálního poškození. Na experimentálních modelech fibrogeneze byla popsána intersticiální infiltrace mononukleárními buňkami a zvýšená transkripce některých cytokinových a chemokinových genů, proto jsme se na studium těchto buněk a molekul zaměřili. Mezi kandidátní geny studované v souvislosti s progresí IgAN patří například geny pro transformující růstový faktor beta-1 (TGF-β1), hepatocytární růstový faktor (HGF), kostní morfogenní protein 7 (BMP7), chemokinový ligand 2, známý jako MCP-1 (monocyte chemotactic protein 1) nebo chemokinový ligand 5, známý jako RANTES (regulated upon activation normal T-cell expressed and secreted).

TGF-β1 je jedním z nejdůležitějších mediátorů expanse mezangiální matrix a je považován za klíčovou komponentu v kaskádě fibrogeneze [11-12]. Přítomnost HGF v renální tkáni může také indukovat zvýšené ukládání matrix, přestože jiné výsledky naznačují možné antifibrotické účinky tohoto faktoru. Zdá se, že výsledný efekt působení HGF na obrat extracelulární matrix je ovlivněn proliferačním stavem, ve kterém se nacházejí cílové renální buňky [13]. Naopak BMP7, který hraje důležitou roli ve vývoji ledvin, se vyznačuje mnoha antifibrogenními vlastnostmi [14]. Také bylo prokázáno, že

zvýšená přítomnost některých pro-zánětlivých a profibrogenních genů, jakými jsou například gen pro MCP-1 nebo RANTES, může být použita jako marker progresivního renálního onemocnění u člověka [15]. Všechny tyto fakty jsme se pokusili ověřit na naší kohortě pečlivě vybraných pacientů nejmodernějšími metodami molekulární biologie. V poslední době je také zvažováno využití sledování infiltrace ledviny zánětlivými buňkami jako markeru progrese chronického renálního poškození, zahrnujícího IgA nefropatii. V některých studiích bylo dokázáno, že infiltrace CD3 pozitivních buněk (T lymfocytů) silně koreluje s intersticiální fibrózou a progresí IgAN [16]. Snahou této práce byl proto také výzkum buněčného infiltrátu a klinických či histologických markerů hrajících roli při vzniku IgA nefropatie a její krátodobé progresi.

Progrese dysfunkce transplantované ledviny

Zatímco v počátcích klinických transplantací byly hlavními problémy zvládnutí akutní rejekce a infekcí, v posledních 15 letech se do popředí pozornosti kliniků i vědců dostala problematika udržení dlouhodobé funkce štěpu [17].

Morfologické změny v transplantované ledvině popisuje Banffská klasifikace [18]. V roce 2005 došlo ke změně diagnostiky chronických změn ve štěpu [19-20], kdy byl z klasifikace eliminován nepřesný výraz CAN (chronic allograft nephropathy, česky byl používán název chronická transplantační nefropatie). V našich pracích publikovaných do roku 2008 je tento výraz dle dřívější definice používán. Za významné alloantigenně závislé faktory pro rozvoj chronického procesu jsou i nadále považovány akutní rejekce a tzv. subklinická rejekce, kdy rejekční změny přetrvávají ve štěpu zdánlivě bez klinické manifestace. Hlavním důvodem chronické dysfunkce transplantované ledviny je ale chronická rejekce. Ta bývá často způsobena protilátkami, ale může být také mediována T lymfocyty. Kromě rejekčních mechanizmů se na progresi dysfunkce transplantované ledviny podílí také alloantigen-nezávislé rizikové faktory, jako snížená kvalita darovaného

orgánu, ischemicko-reperfuzní poškození, infekce, toxicita inhibitorů kalcineurinu (cyklosporinu A nebo takrolimu) nebo hypertenze [18, 21-23].

Diagnostika jednotlivých imunopatogenních stavů po transplantaci ledviny se opírá v současné době hlavně o histologický nález ve tkáni štěpu. Jde o invazivní metodu vyšetření, které má téměř výhradně popisný charakter a poskytuje pouze minimum informací o imunologické aktivitě, ke které ve štěpu dochází a která, jak se v poslední době ukazuje, může mít velice individuální charakter. Sledování imunitních dějů v transplantované ledvině na genové úrovni může být užitečné při hledání nových způsobů predikce individuální prognózy. Stejně jako u onemocnění nativních ledvin i zde může odhalení molekulárních markerů (cytokiny, chemokiny, růstové faktory) rizika vzniku chronických komplikací usnadnit léčbu a prodloužit přežití štěpu.

Tyto cytokiny, chemokiny a růstové faktory jsou produkovány infiltrujícími buňkami, které bývají velice často nalézány v poškozeném štěpu [24-27]. Bylo zjištěno, že sledování genů kódující tyto molekuly a jejich receptory může hrát důležitou roli v monitoraci procesu rejekce [25, 28-29]. Ačkoli patogeneze rejekce je komplexní proces, existuje mnoho kandidátních genů, jejichž zvýšená či snížená aktivita pravděpodobně ovlivňuje individuální rozvoj postižení. U experimentálních modelů hráli roli v patogenezi rejekce štěpu například tumor nekrotizující faktor -alfa (TNF-α), TGF-β1, interferon gama (INF-γ), chemokiny RANTES (CCL5), MCP-1 (CCL2) a jejich receptory CCR5 a CCR2.

Studie akutní rejekce a chronické transplantační nefropatie na zvířecích modelech ukázaly zvýšenou intrarenální expresi *TGF-β1* [30], *TNF-α, IFN-γ, RANTES* a *MCP-1* [31] ve štěpu s rejekcí ve srovnání se zdravými jedinci bez rejekčních příznaků. Na modelu renální intersticiální fibrózy u myší měly *Ccr2* a *Ccr5* knockout zvířata redukovaný vznik fibrózy srovnatelný s neošetřenými jedinci [32-33]. Navíc, zvýšená exprese TGF-β1 u krys vedla k expanzi mezangia, akumulaci proteinů glomerulární matrix a k intersticiální fibróze vedoucí k progresivní glomeruloskleróze [34]. Jedním z cílů této práce bylo ověřit

roli některých genů spojených se zánětem a fibrogenezí v progresi dysfunkce transplantované ledviny.

Ačkoliv je tedy běžně přijímáno, že některými cytokiny zprostředkovaný zánět způsobuje rozvoj buněčného poškození, intersticiální fibrózu a tubulární atrofii s následkem dysfunkce štěpu, už není známo, zda genetická variabilita cytokinů může předurčit možnou náchylnost organizmu k rejekci renálního štěpu. Nedávno byly identifikovány genové polymorfizmy v promotorové oblasti *TNF-α, MCP-1, RANTES* a v překládané oblasti *CCR2, CCR5, IFN-γ* a *TGF-β1*. *In-vitro* studie ukázaly, že některé z nich ovlivňují buď expresi příslušného genu, nebo funkci vzniklého proteinu. *TNF-α* -308G/A a promotorový polymorfizmus -2518A/G v genu *MCP-1* ovlivňují produkci TNF-α [35] a MCP-1 monocyty [36]. RANTES je podobně jako MCP-1 imunoregulační chemokin ovlivňující infiltraci tkáně lymfocyty a makrofágy a haplotyp [-403A; -109C; -28G] v promotoru *RANTES* zvyšuje transkripci tohoto genu [37]. Polymorfizmus *CCR2* +190G/A korespondující se záměnou Val64Ile ve druhém exonu genu zvyšuje transkripci CCR2 [38], zatímco delece 32 párů bazí v kódující oblasti *CCR5* způsobuje posun čtecího rámce a předčasnou terminaci translace v kodonu 185 [39]. IFN-γ je produkován aktivovanými T-lymfocyty a NK buňkami. Aktivuje mononukleární fagocyty a stimuluje buňky k expresi MHC II. i I. třídy. Polymorfizmus *IFN-γ*+874A/T je lokalizován ve vazebném místě transkripčního faktoru NF-κB a způsobuje signifikantní zvýšení transkripce *INF-γ* [40]. Dva polymorfizmy +869T/c a +915G/C v kódující oblasti *TGF-β1* korespondující se záměnou aminokyselin Leu10Pro a Arg25Pro brání produkci TGF-β1 lymfocyty [41].

Vztah polymorfizmů k náchylnosti pacientů k rejekčním epizodám, případně CAN, je sledován v mnoha studiích. Výsledky prací jsou však nejednoznačné a často přináší protichůdné výsledky, což dobře odráží rozhodnutí Banffské klasifikace termín CAN, který není specifickou nemocí, odstranit [19]. Některé práce popisují signifikantní asociaci

polymorfizmů k rejekci štěpu [42-48]. Takové výsledky by mohly vést k identifikaci příjemců ledviny, kteří mají vrozenou predispozici k rejekci štěpu, a na základě této znalosti by bylo možné přizpůsobit imunosupresivní léčbu.

Přístup ke statistickému hodnocení alelických asociačních studií se v posledních letech hodně změnil a také zpřísnil [49-51]. Ve světle těchto faktů se ukázalo, že mnoho studií designovaných „starým“ způsobem, vykazuje často falešně pozitivní výsledky. Hlavním problémem je nízký počet jedinců zařazených do analýzy, který vede ke snížení potřebné statistické síly testu. Jedním z cílů tohoto projektu proto bylo zjistit vliv polymorfizmu vybraných genů na osud transplantované ledviny na základě studia dostatečně velkého, dobře charakterizovaného a reprezentativního souboru nemocných po transplantaci ledviny.

VÝSLEDKY

Vlastní výsledky práce *in extenso* jsou obsaženy v přiložených pracích.

Příloha 1:

BRABCOVÁ, I., KOTSCH, K., HŘIBOVÁ, P., LOUŽECKÁ, A., BARTOŠOVÁ, K., HYKLOVÁ, K., LÁCHA, J., VOLK, H., VIKLICKÝ, O. Intrarenal gene expression of proinflammatory chemokines and cytokines in chronic proteinuric glomerulopathies. Physiological Research, 2007;56(2):221-26.

Příloha 2:

BRABCOVA I., TESAR V., HONSOVA E., LODERÉROVA A., NOVOTNA E., MAIXNEROVA D., MERTA M., BURGELOVA M., HRIBOVA P., SKIBOVA J., ZADRAZIL J., MALY J., VIKLICKY O. Association of advanced vasculopathy and transforming growth factor-beta1 gene expression with immunoglobin A nephropathy progression. Nephrology Dialysis Transplantation, 2010, doi: 10.1093/ndt/gfq423.

Příloha 3:

HŘIBOVÁ, P., LÁCHA, J., KOTSCH, K., VOLK, H.D., BRABCOVÁ, I., SKIBOVÁ, J., VÍTKO, Š., VIKLICKÝ, O. Intrarenal cytokine and chemokine gene expression and kidney graft outcome. Kidney and Blood Pressure Research, 2007;30(5):273-82.

Příloha 4:

BRABCOVÁ, I., PETRÁŠEK, J., HŘIBOVÁ, P., HYKLOVÁ, K., BARTOŠOVÁ, K., LÁCHA, J., VIKLICKÝ, O. Genetic variability of major inflammatory mediators has no impact on the outcome of kidney transplantation. Transplantation, 2007;84(8):1037-44.

KOMENTÁŘ K VÝSLEDKŮM A DISKUZE

I. *Prvním cílem předkládané dizertační páce bylo hodnotit prognostickou roli vybraných molekulárních fenotypů, infiltrace mononukleárních buněk do postižené ledviny a klinických a histologických nálezů u pacientů s IgA nefropatií (přílohy 1 a 2)*

IgAN je postižení charakteristické extrémně variabilním klinickým průběhem, který téměř znemožňuje předpověď dalšího vývoje onemocnění. Progrese IgAN u pacientů se stejným původním morfologickým nálezem v ledvině a podobnou klinickou manifestací onemocnění se v dlouhodobém sledování liší, což naznačuje možnou heterogenitu onemocnění na molekulární úrovni.

V předkládané práci jsme chtěli dokázat, že míra exprese vybraných genů, které hrají roli v regulaci fibrózy a přítomnost některých imunitních buněk v renální tkáni může být asociována s progresí IgA nefropatie. V době diagnózy IgAN jsme detekovali korelaci jak mezi přítomností vybraných genových transkriptů tak i přítomností zánětlivého infiltrátu se známými rizikovými faktory progrese onemocnění jakými jsou snížená renální funkce nebo proteinurie. Nicméně ve dvouletém sledování, vedle klinických parametrů, závisela progrese IgAN jen na závažnosti chronické vaskulopatie a na síle exprese genu pro TGF-β1.

Chronická vaskulopatie jako důsledek hypertenze a stárnutí způsobuje renální ischemii. Následkem renální ischémie dochází k angiotensin II závislému zvýšení exprese TGF-β1, které bylo identifikováno jako kritické pro rozvoj renální fibrogeneze [52]. *TGF-β1,* často spojovaný s regulací buněčné proliferace, hypertrofií, apoptózou a fibrogenezí je také centrálním stimulem dějů vedoucích k chronickému progresivnímu renálnímu onemocnění. Tento růstový faktor dále jako hlavní regulátor ovlivňuje mnoha cestami produkci a degradaci extracelulární matrix (kolagen, fibronektin, proteoglykany), zvyšuje expresi integrinů a snižuje aktivitu proteáz degradujících matrix [53]. Navíc byla

v nedávné době prokázána prediktivní schopnost také dalších mRNA a proteinů signalizační kaskády TGF-β1 na vývoj a progresi CKD (chronic kidney desease) jak u myši, tak u člověka [54]. Tato pozorování se shodují s výsledky předkládané práce podobně jako studie Katafuschi et al [55], kde byla sledována role vaskulárních onemocnění při progresi IgAN u 71 pacientů. Ukázalo se, že přítomnost cévnatých oblastí je stejně důležitým prediktorem glomerulární sklerózy a progrese IgAN jako hypertenze.

Další charakteristikou hodnocenou v souvislosti se závažností renálního poškození a progresivního renálního selhání je intersticiální zánět. V předkládané práci jsme prokázali, že intersticiální infiltrace T a B lymfocyty spolu s makrofágy koreluje v době biopsie se zvýšenou expresí genů pro chemokiny, které atrahují lymfocyty a monocyty do místa poškození. Tyto buňky tedy prokazatelně hrají aktivní roli ve fibrogenezi [53]. Při dlouhodobém sledování se ale ukázalo, že infiltrace makrofágy, ne však lymfocyty je prediktivní pro rozvoj a progresi IgAN. Tyto výsledky jsou v konstrastu s výsledky retrospektivních studií, ve kterých přítomnost tubulointersticiálního zánětu určovala prognózu IgAN [16, 56]. V těchto studiích však nikdy nebyly zveřejněny relevantní informace o imunosupresivní léčbě pacientů. V naší studii nebyl prokázán statisticky významný rozdíl v imunosupresivní léčbě mezi skupinou „progresorů“ a „neprogresorů“.

Všeobecně přijímanou známkou progrese IgAN je také proteinurie [57] a v naší práci jsme prokázali vztah mezi proteinurií a genovou expresí některých chemokinů a růstového faktoru HGF.

Kromě známých rizikových faktorů progrese IgAN jakými jsou vyšší proteinurie nebo snížená renální funkce se v naší studii tedy podařilo prokázat, že pokročilá vaskulopatie a molekulární markery fibrogeneze jsou asociovány s krátkodobou progresí IgAN dokonce lépe než známé klinické rizikové faktory nebo infiltrace štěpu T a B lymfocyty. Toto pozorování tak přispívá ke změně představy o vzniku a vývoji IgAN jako o imunitními buňkami zprostředkovaném onemocnění, jehož progresivní formy je

nezbytné léčit imunosupresí založenou na blokádě funkce T lymfocytů. Nicméně aby bylo možné upravovat klinická rozhodnutí na základě měření molekulárních fenotypů dle našich výsledku získaných na limitovaném počtu pacientů, bylo by nutné je ověřit v multi-centrické prospektivní studii s delším časovým sledováním po diagnostické biopsii.

II. *Druhým cílem předkládané práce bylo hodnotit intrarenální genové exprese vybraných cytokinů a chemokinů u pacientů po transplantaci ledviny s diagnostikovanou chronickou transplantační nefropatií (podle Banffské klasifikace do roku 2005) (příloha 3)*

Chronická transplantační nefropatie podle starší klasifikace byla detekována ve většině protokolárních biopsií prováděných ve 12. měsíci po transplantaci [58]. Podobně jako u onemocnění vlastních ledvin je proteinurie považována za důležitý rizikový faktor selhání ledvinného štěpu [59-61]. Cílem další části předkládané práce byla detekce intrarenálních mRNA pro růstové faktory, cytokiny a chemokiny a jejich korelace s klinickými a histomorfologickými daty po transplantaci ledviny.

Podobně jako u IgA nefropatie i v biopsiích pacientů s diagnostikovanou CAN jsme nalezli asociaci mezi intrarenální upregulací exprese genu pro TGF-β1, MCP-1 a zhoršenou dlouhodobou funkcí štěpu a zkrácením přežití transplantátu. V době biopsie měli pacienti s CAN významně upregulovány mRNA dalších sledovaných genů (*IL-10, TGF-β1, IL-6, MCP-1, RANTES* a *TNF-α*) oproti kontrolní skupině s normálním nálezem. Dále jsme zjistili, že intrarenální exprese všech sledovaných cytokinů a chemokinů při CAN koreluje s výší proteinurie. Nabízí se tedy hypotéza, že proteinurie a ultrafiltrace růstových faktorů způsobují upregulaci zánětlivých cytokinů a dalších růstových faktorů, což vede k intersticiální fibróze transplantované ledviny. Studium histomorfologických dat ukázalo že tubulitida štěpu postiženého CAN koreluje s intrarenální expresí chemokinů MCP-1 a RANTES. Nezjistili jsme korelaci intrarenální exprese *TGF-β1* mRNA

s intersticiální fibrózou, glomerulopatií, tubulární atrofií ani fibrotickým ztluštěním intimy cév. Vyšší exprese *TGF-β1* byla však zaznamenána ve štěpech s přítomností mírné tubulitidy (dle Banffské klasifikace 1997). Tyto skutečnosti potvrzují náš předpoklad, že pod diagnostickým pojmem CAN jsou zahrnuty štěpy s různou imunologickou aktivitou. Aktivitu onemocnění naznačuje přítomnost tubulitidy a zároveň zvýšená exprese genů pro některé cytokiny a chemokiny. Na základě výše uvedených údajů lze doporučit pečlivé sledování pacientů po transplantaci ledviny, kteří mají v ledvinném štěpu zvýšenou expresi prozánětlivých genů. Také proteinurie se jeví jako významný modifikující faktor, který vyžaduje terapeutický zásah.

III. Třetím cílem předkládané práce bylo najít vztah mezi vybranými genovými polymorfizmy cytokinů a chemokinů na osud transplantované ledviny (příloha 4)

Expresní genová analýza, která byla použita ve výše diskutovaných pracích je prováděna v bioptické renální tkáni. Jde tedy o invazivní metodu, kterou nelze použít pro preventivní odhad rizikových pacientů. V posledních letech se obrovské úsilí věnovalo studiu genových polymorfizmů a vytváření genotypových profilů různých onemocnění. Z dostupné literatury bylo možné předpokládat, že k identifikaci jedinců predisponovaných k vyšší expresi cytokinů nebo chemokinů a tím k vyššímu riziku dysfunkce štěpu by mohla napomoci právě analýza polymorfizmů jejich genů. Šlo by o neinvazivní metodu detekce z periferní krve pacienta odebrané v jakémkoli období jeho života. V minulosti bylo publikováno mnoho studií zabývajících se asociací genových polymorfizmů s akutní nebo chronickou rejekcí. Jejich výsledky jsou však nejednoznačné nebo dokonce protichůdné.

V naší práci jsme hodnotili pouze ty cytokiny, jejichž role v patogenezi rejekce byla potvrzena na zvířecích modelech a polymorfizmy, jejichž vliv na expresi daného genu nebo funkci finálního proteinu potvrdily *in-vitro* studie. Nicméně na rozdíl od dříve

publikovaných prací [42, 48, 62-63] jsme nedetekovali žádnou asociaci mezi vybranými polymorfizmy a rizikem akutní, subklinické rejekce nebo CAN. Je pro to několik vysvětlení, která se odvolávají na v současnosti uznávaná pravidla a předpoklady, které má alelická asociační studie splňovat [49, 51] a kterých se dřívější studie striktně nedržely. Ve světle těchto faktů se ukázalo, že mnoho studií designovaných „starým“ způsobem vykazuje často falešně pozitivní výsledky.

Výběr kandidátních genů při designování alelické asociační studie by mělo vycházet z detailního porozumění patofyziologických dějů probíhajících v ledvině a na základě této znalosti by se mělo přistupovat k interpretaci výsledků. Polymorfizmy v genech cytokinů účastnících se patofyziologických dějů probíhajících při rejekcích štěpu jsou studovány v mnoha světových pracích. Nicméně například ve studii Sankaran at al [42] byli jedinci predisponovaní k vysoké produkci TNF-α a IL-10 ve zvýšeném riziku akutní rejekce štěpu, ačkoli IL-10 je znám jako antizánětlivý cytokin, který u transgenních myších modelů inhibuje produkci TNF-α. Dále je vhodné testovat některé funkčně relevaltní geny společně, protože takový přístup zvyšuje šanci na detekci rizikového faktoru. Nicméně je potřeba si uvědomit, že znásobení počtu porovnávání vede k falešně pozitivním výsledkům [64] a to je nutné ošetřit při statistickém zpracování vhodnou korekcí na mnohočetné testování. Taková korekce například ukázala, že asociace mezi alelami *TNF-α* -308A, *TGF-β1* +915G a *IL-10* -1082G a akutní rejekcí štěpu nalezená ve studii Alakuppi at al [62] je falešně pozitivní. Do studií by měli být zařazováni jedinci z homogenního souboru nemocných co se týče například léčby a dalšího ošetření [65]. Vliv vrozené predispozice na incidenci rejekce může být maskován užíváním různé imunosuprese a výsledky mohou opět být mylně pozitivně interpretovány [48]. Oproti jiným pracím [63] byla spolehlivost genotypových analýz v naší studii pečlivě testována a je patrné že výsledky naměřené u kontrolní skupiny splňují Hardy-Weinbergerovo kritérium a frekvence genotypů jsou v souladu s referenční HapMap databází.

Dalším, nejzřejmějším vysvětlením rozdílu výsledků naší a předchozích studií je nedostatečná statistická síla testů použitých v těchto studiích. Kvalita alelické asociační studie je dána především počtem vzorků zařazených do analýz [51], který by měl být určen z výpočtu statistické síly ještě ve fázi designování studie. Navíc by především studie s malým počtem vzorků měly zahrnovat validaci výsledků na nezávislé kohortě pacientů.

V naší studii žádný z funkčně relevantních polymorfizmů v genech *TNF-a*, *MCP1*, *RATES*, *CCR2*, *CCR5*, *IFN-γ* a *TGF-β1* nezvyšoval riziko subklinické rejekce, akutní rejekce nebo CAN u sledovaných pacientů. Přestože není pochyb že Th1 a Th2 imunitní reakce hraje roli v patogenezi rejekce štěpu, vrozené predispozice k akutní rejekci nebo CAN u bílých pacientů nebyly zatím jednoznačně nalezeny. Je možné, že proces rejekce štěpu je určován kombinací mnoha genových polymorfizmů, které alelická asociační studie neodhalí, popřípadě mohou existovat další, zatím nesledované mutace které mají zásadní vliv na vznik potransplantačních komplikací. Identifikace takových polymorfizmů a studie, které budou plně splňovat v současnosti uznávané standardy pro design alelických asociačních studií můžou vést k detekci rizikových pacientů, úpravě léčby a významně lepšímu přežití štěpu.

ZÁVĚR

V předkládané dizertační práci jsou shrnuty výsledky studií provedených v Transplantační laboratoři a na Klinice nefrologie Institutu klinické a experimentální medicíny v Praze, kde jsme se zabývali analýzou molekulárně – genetických faktorů ovlivňujících progresi IgA nefropatie a dysfunkci transplantované ledviny. Snahou všech studií bylo nalezení markerů, které by pomohli včas identifikovat pacienty ve zvýšeném riziku progrese onemocnění. Podařilo se nám prokázat že:

1. V případě IgA nefropatie aktivita sledovaných genů i infiltrace mononukleárních buněk do renální tkáně v době biopsie koreluje s obecně uznávanými klinickými faktory podporujícími progresi IgA nefropatie. Dvouleté sledování ale ukázalo, že pokročilá vaskulopatie, up-regulace transformujícího růstového faktoru *TGF-β1* a infiltrace tkáně makrofágy je asociována s dvouletou progresí IgAN lépe, než známé klinické rizikové faktory jakými jsou vyšší proteinurie, snížená renální funkce nebo infiltrace štěpu T a B lymfocyty. Toto pozorování tak přispívá ke změně představy o vzniku a vývoji IgAN jako o imunitními buňkami zprostředkovaném onemocnění, jehož progresivní formy je nezbytné léčit imunosupresí založenou na blokádě funkce T lymfocytů (*přílohy 1 a 2*).

2. Podobně jako u IgA nefropatie i v iniciálních biopsiích pacientů po transplantaci ledviny s diagnostikovanou CAN je asociace mezi intrarenální upregulací exprese genu pro TGF-β1, ale také pro MCP-1 a zhoršenou dlouhodobou funkcí štěpu a zkrácenou dobou přežití štěpu. V průběhu akutní rejekce i dalších poškození štěpu jsme detekovali v různé míře up-regulovány cytokiny a chemokiny *IL-10, TGF-β1, IL-6, MCP-1, RANTES* a *TNF-α.* Profil intrarenální exprese těchto genů se liší během dysfunkce štěpu z různých příčin. Úroveň jejich exprese nelze využít k diagnostickým účelům,

může ale upozornit na vyšší imunologickou aktivitu v ledvinném štěpu, která může vést k rychlejšímu selhání renální funkce (*příloha 3*).

3. Žádný z funkčně relevantních polymorfizmů v genech *TNF-a, MCP-1, RANTES, CCR2, CCR5, IFN-γ* a *TGF-β1* nezvyšuje riziko subklinické rejekce, akutní rejekce nebo CAN u sledovaných pacientů, což odráží komplexnost imunitní odpovědi proti alloantigenu. Je pravděpodobné, že genetická predispozice k rejekci štěpu je určena mnoha genovými polymorfizmy, přičemž individuální alely přispívají k výslednému fenotypu pouze nevýrazně a alelickou asociační studií nelze takovýto příspěvek detekovat (*příloha 4*).

Závěrem lze konstatovat, že tato studie přispívá k identifikaci nových biomarkerů, pomocí kterých lze identifikovat nemocné v riziku progrese IgA nefropatie a komplikací po transplantaci ledviny.

LITERATURA

1. *URL: <http://www.nefrol.cz/> [cit. 2010-11-1]*.
2. Rychlik, I., et al., *The Czech registry of renal biopsies. Occurrence of renal diseases in the years 1994-2000.* Nephrol Dial Transplant, 2004. **19**(12): p. 3040-9.
3. Remuzzi, G. and T. Bertani, *Pathophysiology of progressive nephropathies.* N Engl J Med, 1998. **339**(20): p. 1448-56.
4. D'Amico, G., *Natural history of idiopathic IgA nephropathy and factors predictive of disease outcome.* Semin Nephrol, 2004. **24**(3): p. 179-96.
5. Donadio, J.V., et al., *Proteinuria patterns and their association with subsequent end-stage renal disease in IgA nephropathy.* Nephrol Dial Transplant, 2002. **17**(7): p. 1197-203.
6. Ibels, L.S. and A.Z. Gyory, *IgA nephropathy: analysis of the natural history, important factors in the progression of renal disease, and a review of the literature.* Medicine (Baltimore), 1994. **73**(2): p. 79-102.
7. Woo, K.T., et al., *The natural history of IgA nephritis in Singapore.* Clin Nephrol, 1986. **25**(1): p. 15-21.
8. Reich, H.N., et al., *Remission of proteinuria improves prognosis in IgA nephropathy.* J Am Soc Nephrol, 2007. **18**(12): p. 3177-83.
9. Julian, B.A. and J. Novak, *IgA nephropathy: an update.* Curr Opin Nephrol Hypertens, 2004. **13**(2): p. 171-9.
10. D'Amico, G., *Natural history of idiopathic IgA nephropathy: role of clinical and histological prognostic factors.* Am J Kidney Dis, 2000. **36**(2): p. 227-37.
11. Isaka, Y., et al., *Glomerulosclerosis induced by in vivo transfection of transforming growth factor-beta or platelet-derived growth factor gene into the rat kidney.* J Clin Invest, 1993. **92**(6): p. 2597-601.
12. Schnaper, H.W., et al., *TGF-beta signal transduction in chronic kidney disease.* Front Biosci, 2009. **14**: p. 2448-65.
13. Esposito, C., et al., *The antifibrogenic effect of hepatocyte growth factor (HGF) on renal tubular (HK-2) cells is dependent on cell growth.* Growth Factors, 2009. **27**: p. 137-180.
14. Sugimoto, H., et al., *Renal fibrosis and glomerulosclerosis in a new mouse model of diabetic nephropathy and its regression by bone morphogenic protein-7 and advanced glycation end product inhibitors.* Diabetes, 2007. **56**(7): p. 1825-33.

15. Mezzano, S.A., et al., *Tubular NF-kappaB and AP-1 activation in human proteinuric renal disease.* Kidney Int, 2001. **60**(4): p. 1366-77.
16. Myllymaki, J.M., et al., *Severity of tubulointerstitial inflammation and prognosis in immunoglobulin A nephropathy.* Kidney Int, 2007. **71**(4): p. 343-8.
17. Cho, Y.W. and P.I. Terasaki, *Long-term survival.* Clin Transpl, 1988: p. 277-82.
18. Racusen, L.C., et al., *The Banff 97 working classification of renal allograft pathology.* Kidney Int, 1999. **55**(2): p. 713-23.
19. Solez, K., et al., *Banff '05 Meeting Report: differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN').* Am J Transplant, 2007. **7**(3): p. 518-26.
20. Solez, K., et al., *Banff 07 classification of renal allograft pathology: updates and future directions.* Am J Transplant, 2008. **8**(4): p. 753-60.
21. Carpenter, C.B., *Long-term failure of renal transplants: adding insult to injury.* Kidney Int Suppl, 1995. **50**: p. S40-4.
22. Paul, L.C., *Chronic renal transplant loss.* Kidney Int, 1995. **47**(6): p. 1491-9.
23. Solez, K., et al., *International standardization of criteria for the histologic diagnosis of renal allograft rejection: the Banff working classification of kidney transplant pathology.* Kidney Int, 1993. **44**(2): p. 411-22.
24. Adams, A.B., et al., *The role of TNF receptor and TNF superfamily molecules in organ transplantation.* Am J Transplant, 2002. **2**(1): p. 12-8.
25. Baan, C.C. and W. Weimar, *Intragraft cytokine gene expression: implications for clinical transplantation.* Transpl Int, 1998. **11**(3): p. 169-80.
26. Border, W.A. and N.A. Noble, *Transforming growth factor beta in tissue fibrosis.* N Engl J Med, 1994. **331**(19): p. 1286-92.
27. Noronha, I.L., et al., *In situ expression of tumor necrosis factor-alpha, interferon-gamma, and interleukin-2 receptors in renal allograft biopsies.* Transplantation, 1992. **54**(6): p. 1017-24.
28. Waller, J.R. and M.L. Nicholson, *Molecular mechanisms of renal allograft fibrosis.* Br J Surg, 2001. **88**(11): p. 1429-41.
29. Khan, F., S. Agrawal, and S. Agrawal, *Genetic predisposition and renal allograft failure: implication of non-HLA genetic variants.* Mol Diagn Ther, 2006. **10**(4): p. 205-19.
30. Paul, L.C., et al., *Growth factor transcripts in rat renal transplants.* Am J Kidney Dis, 1996. **28**(3): p. 441-50.

31. Nadeau, K.C., H. Azuma, and N.L. Tilney, *Sequential cytokine dynamics in chronic rejection of rat renal allografts: roles for cytokines RANTES and MCP-1.* Proc Natl Acad Sci U S A, 1995. **92**(19): p. 8729-33.
32. Kitagawa, K., et al., *Blockade of CCR2 ameliorates progressive fibrosis in kidney.* Am J Pathol, 2004. **165**(1): p. 237-46.
33. Eis, V., et al., *Chemokine receptor CCR1 but not CCR5 mediates leukocyte recruitment and subsequent renal fibrosis after unilateral ureteral obstruction.* J Am Soc Nephrol, 2004. **15**(2): p. 337-47.
34. Sanderson, N., et al., *Hepatic expression of mature transforming growth factor beta 1 in transgenic mice results in multiple tissue lesions.* Proc Natl Acad Sci U S A, 1995. **92**(7): p. 2572-6.
35. Wilson, A.G., et al., *Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation.* Proc Natl Acad Sci U S A, 1997. **94**(7): p. 3195-9.
36. Rovin, B.H., L. Lu, and R. Saxena, *A novel polymorphism in the MCP-1 gene regulatory region that influences MCP-1 expression.* Biochem Biophys Res Commun, 1999. **259**(2): p. 344-8.
37. Guignard, F., et al., *Gene organization and promoter function for CC chemokine receptor 5 (CCR5).* J Immunol, 1998. **160**(2): p. 985-92.
38. Nakayama, E.E., et al., *A CCR2-V64I polymorphism affects stability of CCR2A isoform.* Aids, 2004. **18**(5): p. 729-38.
39. Samson, M., et al., *Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene.* Nature, 1996. **382**(6593): p. 722-5.
40. Rossouw, M., et al., *Association between tuberculosis and a polymorphic NFkappaB binding site in the interferon gamma gene.* Lancet, 2003. **361**(9372): p. 1871-2.
41. Awad, M.R., et al., *Genotypic variation in the transforming growth factor-beta1 gene: association with transforming growth factor-beta1 production, fibrotic lung disease, and graft fibrosis after lung transplantation.* Transplantation, 1998. **66**(8): p. 1014-20.
42. Sankaran, D., et al., *Cytokine gene polymorphisms predict acute graft rejection following renal transplantation.* Kidney Int, 1999. **56**(1): p. 281-8.

43. Pelletier, R., et al., *Evidence for a genetic predisposition towards acute rejection after kidney and simultaneous kidney-pancreas transplantation.* Transplantation, 2000. **70**(4): p. 674-80.
44. Kruger, B., et al., *A Monocyte chemoattractant protein-1 (MCP-1) polymorphism and outcome after renal transplantation.* J Am Soc Nephrol, 2002. **13**(10): p. 2585-9.
45. Abdi, R., et al., *Chemokine receptor polymorphism and risk of acute rejection in human renal transplantation.* J Am Soc Nephrol, 2002. **13**(3): p. 754-8.
46. Fischereder, M., et al., *CC chemokine receptor 5 and renal-transplant survival.* Lancet, 2001. **357**(9270): p. 1758-61.
47. McDaniel, D.O., et al., *Combined analysis of cytokine genotype polymorphism and the level of expression with allograft function in African-American renal transplant patients.* Transpl Immunol, 2003. **11**(1): p. 107-19.
48. Asderakis, A., et al., *Association of polymorphisms in the human interferon-gamma and interleukin-10 gene with acute and chronic kidney transplant outcome: the cytokine effect on transplantation.* Transplantation, 2001. **71**(5): p. 674-7.
49. Cooper, D.N., R.L. Nussbaum, and M. Krawczak, *Proposed guidelines for papers describing DNA polymorphism-disease associations.* Hum Genet, 2002. **110**(3): p. 207-8.
50. Balding, D.J., *A tutorial on statistical methods for population association studies.* Nat Rev Genet, 2006. **7**(10): p. 781-91.
51. Hattersley, A.T. and M.I. McCarthy, *What makes a good genetic association study?* Lancet, 2005. **366**(9493): p. 1315-23.
52. Border, W.A. and N.A. Noble, *Interactions of transforming growth factor-beta and angiotensin II in renal fibrosis.* Hypertension, 1998. **31**(1 Pt 2): p. 181-8.
53. Wynn, T.A., *Cellular and molecular mechanisms of fibrosis.* J Pathol, 2008. **214**(2): p. 199-210.
54. Ju, W., et al., *Renal gene and protein expression signatures for prediction of kidney disease progression.* Am J Pathol, 2009. **174**(6): p. 2073-85.
55. Katafuchi, R., et al., *Microvascular disease and the progression of IgA nephropathy.* Am J Kidney Dis, 1990. **15**(1): p. 72-9.
56. Van Es, L.A., et al., *GMP-17-positive T-lymphocytes in renal tubules predict progression in early stages of IgA nephropathy.* Kidney Int, 2008. **73**(12): p. 1426-33.

57. Jafar, T.H., et al., *Progression of chronic kidney disease: the role of blood pressure control, proteinuria, and angiotensin-converting enzyme inhibition: a patient-level meta-analysis.* Ann Intern Med, 2003. **139**(4): p. 244-52.
58. Nankivell, B.J., et al., *The natural history of chronic allograft nephropathy.* N Engl J Med, 2003. **349**(24): p. 2326-33.
59. Arias, M., et al., *Non-immunologic intervention in chronic allograft nephropathy.* Kidney Int Suppl, 2005(99): p. S118-23.
60. Joosten, S.A., et al., *Chronic renal allograft rejection: pathophysiologic considerations.* Kidney Int, 2005. **68**(1): p. 1-13.
61. Massy, Z.A., et al., *Chronic renal allograft rejection: immunologic and nonimmunologic risk factors.* Kidney Int, 1996. **49**(2): p. 518-24.
62. Alakulppi, N.S., et al., *Cytokine gene polymorphisms and risks of acute rejection and delayed graft function after kidney transplantation.* Transplantation, 2004. **78**(10): p. 1422-8.
63. Dmitrienko, S., et al., *Immune response gene polymorphisms in renal transplant recipients.* Transplantation, 2005. **80**(12): p. 1773-82.
64. Altman DG, M.D., Bryant TN, Gardner MJ, eds, *Statistics with confidence.* BJM Books, 2000. **page 163**.
65. Miettinen, O.S. and E.F. Cook, *Confounding: essence and detection.* Am J Epidemiol, 1981. **114**(4): p. 593-603.

PŘÍLOHY

Příloha 1:

BRABCOVÁ, I., KOTSCH, K., HŘIBOVÁ, P., LOUŽECKÁ, A., BARTOŠOVÁ, K., HYKLOVÁ, K., LÁCHA, J., VOLK, H., VIKLICKÝ, O. Intrarenal gene expression of proinflammatory chemokines and cytokines in chronic proteinuric glomerulopathies. Physiological Research, 2007;56(2):221-26.

Příloha 2:

BRABCOVA I., TESAR V., HONSOVA E., LODERÉROVA A., NOVOTNA E., MAIXNEROVA D., MERTA M., BURGELOVA M., HRIBOVA P., SKIBOVA J., ZADRAZIL J., MALY J., VIKLICKY O. Association of advanced vasculopathy and transforming growth factor-beta1 gene expression with immunoglobin A nephropathy progression. Nephrology Dialysis Transplantation, 2010, doi: 10.1093/ndt/gfq423.

Příloha 3:

HŘIBOVÁ, P., LÁCHA, J., KOTSCH, K., VOLK, H.D., BRABCOVÁ, I., SKIBOVÁ, J., VÍTKO, Š., VIKLICKÝ, O. Intrarenal cytokine and chemokine gene expression and kidney graft outcome. Kidney and Blood Pressure Research, 2007;30(5):273-82.

Příloha 4:

BRABCOVÁ, I., PETRÁŠEK, J., HŘIBOVÁ, P., HYKLOVÁ, K., BARTOŠOVÁ, K., LÁCHA, J., VIKLICKÝ, O. Genetic variability of major inflammatory mediators has no impact on the outcome of kidney transplantation. Transplantation, 2007;84(8):1037-44.

PŘÍLOHA 1

Intrarenal Gene Expression of Proinflammatory Chemokines and Cytokines in Chronic Proteinuric Glomerulopathies

I. BRABCOVÁ[1], K. KOTSCH[3], P. HŘIBOVÁ[1], A. LOUŽECKÁ[1], K. BARTOŠOVÁ[2], K. HYKLOVÁ[1], J. LÁCHA[1,2], H.-D. VOLK[3], O. VIKLICKÝ[1,2]

Transplant Laboratory[1], Department of Nephrology[2], Institute for Clinical and Experimental Medicine, Prague, Czech Republic and Institute of Medical Immunology[3], Medical University Charité, Humboldt University Berlin, Germany

Received January 2, 2006
Accepted March 14, 2006
On-line available March 23, 2006

Summary

Proteinuria has been recently shown to be an independent risk factor for the progression of chronic nephropathies, but the actual mechanisms by which urinary protein load damages renal tissue in humans remain unsolved. Using real-time RT-PCR method we evaluated intrarenal mRNA expression of various cytokines and chemokines in patients with biopsy-proven IgA nephropathy (IgAN, n=11), membranous nephropathy (MN, n=6) and focal and segmental glomerulosclerosis (FSGS, n=6) who exhibited proteinuria over 0.5 g/day. There was a significant positive correlation between the proteinuria extent and the intrarenal RANTES (regulated upon activation normal T cell expressed and secreted) mRNA expression in patients with IgAN, a similar trend was also observed in patients with MN and FSGS. There were no clear relationships between the proteinuria and intrarenal mRNA expression of tumor necrosis factor α, transforming growth factor β1 and monocyte chemoattractant peptide-1. There were no differences in the pattern of cytokine mRNA expression between different glomerulopathies. In conclusion, our results support the hypothesis that lymphocytes, macrophages and their products provoke tissue injury in response to proteinuria independently of the nature of renal diseases in man.

Key words
Chemokines • Gene expression • Glomerulonephritis • Proteinuria

Introduction

Chronic glomerulopathies represent an important cause of end-stage renal diseases especially among younger patients. Despite a poor understanding of their pathogenesis, different morphological findings and different treatment modalities, the disease progression shares many similarities. Both hypertension and proteinuria represent the independent risk factors for the progression of the disease (Remuzzi and Bertani 1998, Hirschberg and Wang 2005). There is growing evidence suggesting that proteinuria determines tubular cell injury and activation. As a result of this injury, nuclear factor kappa B (NF-κB) translocates to the nucleus of tubular cells, binds to the specific DNA sequence and enhances gene transcription and generation inflammatory cytokines

Fax +420 241 062 164
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

Table 1. Clinical parameters at the time of renal biopsy.

Nephropathy	IgAN	FSGS	MN
n (M/F)	11 (6/5)	6 (3/3)	6 (1/5)
Age (years)	40.3 [25.2-54.3]	43.6 [26.9-62.6]	66 [35-76.4]
BMI (kg/m^2)	27.9 [20.1-40.2]	25.0 [18.4-28.0]	24.0 [17.5-33.2]
Total cholesterol (mmol/l)	6.4 [4.6-7.6]	9.4 [6.3-15.0]	8.0 [4.88-17.2]
Serum creatinine (μmol/l)	145 [67-617]	85 [58-140]	111.8 [60-150]
Serum albumin (g/l)	38.7 [31.2-42.1]	31.5 [19.3-47.1]	25.4 [14 - 33]
GFR (ml/s)	1.0 [0.2-2.8]	1.5 [0.7-3.0]	0.9 [0.5 – 2.0]
Proteinuria (g/day)	2.1 [0.5-6.0]	5.3 [2.1-13.3]	6.5 [3.5-3]
Hypertension (%)	54.5	33.3	66.7

Data are shown as median [minimum-maximum]. GFR - Glomerular filtration rate estimated using the Cockcroft-Gault formula, IgAN – IgA nephropathy, FSGS – focal and segmental glomerulosclerosis, MN – membranous nephropathy, BMI – body mass index. Hypertension was defined as the presence of antihypertensive therapy or blood pressure >140/90 mm Hg before biopsy was performed.

and chemokines (Mezzano *et al.* 2001) such as monocyte chemoattractant peptide-1 (MCP-1), regulated upon activation of normal T-cell expressed and secreted (RANTES), and intracellular cell adhesion molecule-1 (ICAM-1) (Schlondorff 1995). Locally secreted immunoregulating chemokines mediate enhanced tissue infiltration with lymphocytes and monocytes and this process seems to influence the disease progression (Ootaka *et al.* 1995, Watanabe *et al.* 2001, Ikezumi *et al.* 2004). Since most studies on this topic come from experimental models and experience in human kidney biopsies has not been published so far, we studied the gene expression of proinflammatory cytokines and chemokines in patients with primary proteinuric glomerulopathies.

Patients and Methods

Patients

Experiments were performed on 34 patients who were referred to our center from May 2002 to January 2004 because of proteinuria and in whom renal biopsy proved primary chronic glomerulopathy. Eleven patients suffered from IgAN, six patients from FSGS and six others from MN. Eleven patients had other morphological findings and were not included in the study. Clinical parameters obtained from patients at the time of biopsy are shown in Table 1. All patients gave their written informed consent to participate in the study and the study protocol was approved by the Ethical Committee of the Institute for Clinical and Experimental Medicine in Prague.

Renal biopsy

All biopsies were performed using a tru-cut needle (Uni-Cut Nadeln, Angiomed) guided by ultrasound (Toshiba, Power Vision 6000). Most of the renal tissue was used for routine histology. Small portions (~2 mm) of the cortical or juxtamedulary zone of the renal tissue were immediately frozen in liquid nitrogen and stored at –80 °C. For the analysis, samples with at least one glomerulus were used. Biopsies were performed to evaluate primary diagnosis and the patients were thus not under immunosuppressive therapy at the time of biopsy.

RNA isolation and real-time quantitative RT-PCR

After renal tissue had been homogenized and total RNA extracted using StrataPrep Total RNA Microprep Kit (Stratagene, La Jolla, United States), it was reversely transcribed into complementary DNA (cDNA), as described elsewhere (Platzer *et al.* 1994). Complementary DNA was amplified by real-time quantitative polymerase chain reaction (PCR) (TaqMan®, ABI Prism 5700 Sequence Detection System, Perkin Elmer, Darmstadt, Germany) using fluorogenic probes. Messenger RNA of chemokines (MCP-1, RANTES) and cytokines – tumor necrosis factor α (TNF-α) and transforming growth factor β1 (TGF-β1) – was quantified. The HPRT housekeeping gene (hypoxanthin-guanin-phosphoribosyl-transferase) was used as an internal standard in the comparative threshold cycle method ($2^{-\Delta Ct}$). All investigated mRNAs were measured in duplicates for each sample. All primers and

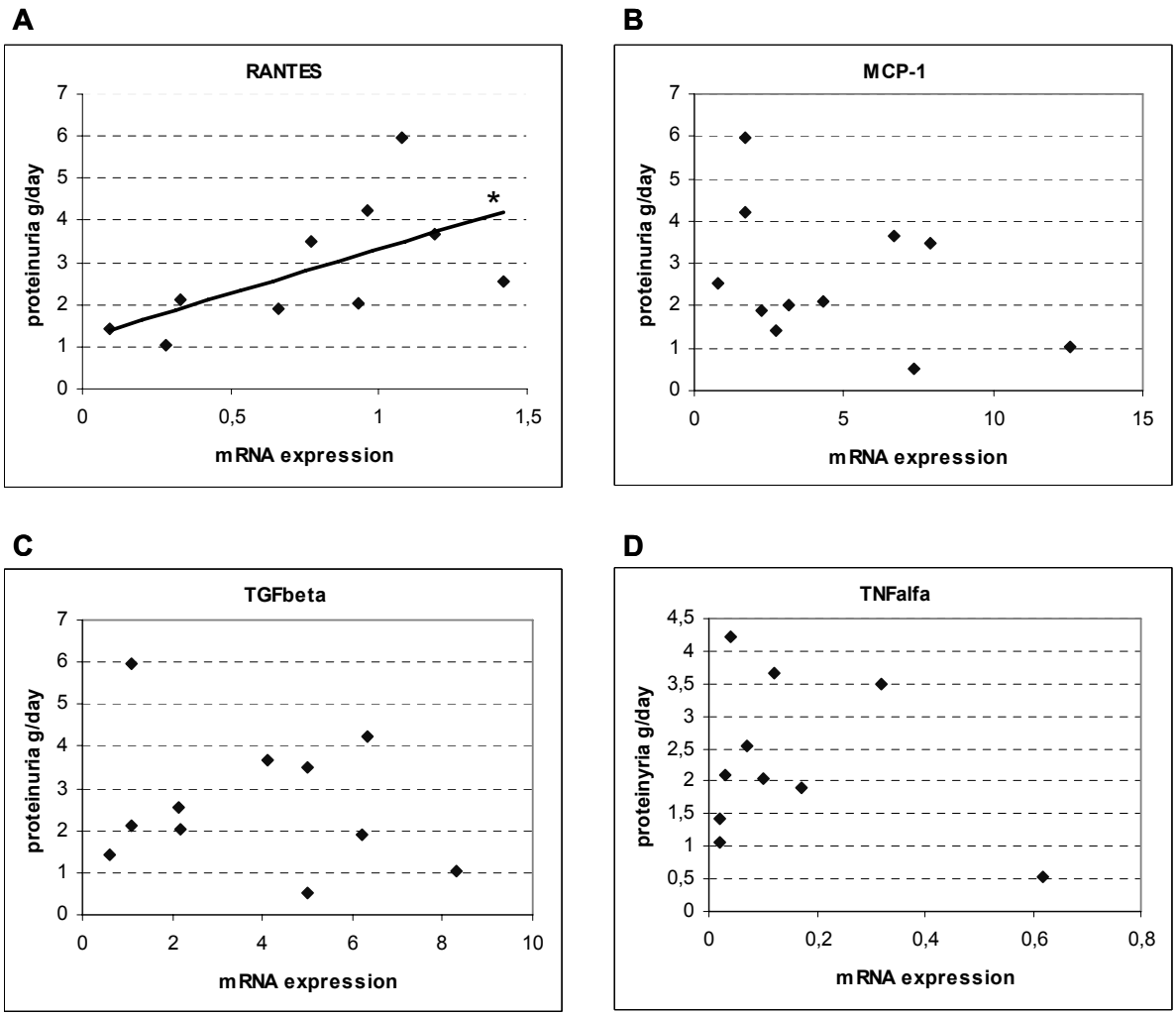

Fig. 1. Correlation between the mRNA expression of cytokine and chemokine genes (normalized to HPRT, $2^{-\Delta Ct}$ method) and the proteinuria in patients with proven IgA nephropathy (IgAN). There was a significant relationships between the intrarenal RANTES mRNA expression and the proteinuria (a). *= $p<0.05$; n=10. Correlations between MCP-1, TGF-β and TNF-α intrarenal gene expressions and proteinuria have not reached statistical significance.

probes were designed, and assays validated at the Institute of Medical Immunology, Universitätsmedizin Charité, Berlin. Because preceding experiments demonstrated amplification efficiencies in our system of nearly 1 for all panels, specific gene expression was calculated relative to that of the housekeeping gene HPRT. Samples were considered negative if the Ct values exceeded 40 cycles.

Statistical analysis

Relationship between proteinuria and mRNA expression was assessed using Spearman correlation coefficient calculation. $P<0.05$ value was accepted to be statistically significant. For the comparison of clinical groups the Kruskal-Wallis test was used.

Results

In general, the mean age of patients diagnosed with MN was higher than the age of patients with other diagnosis and patients with MN had higher prevalence of hypertension. Both patient groups, with MN and FSGS, had higher proteinuria and cholesterol plasma levels than patients with IgAN because of the presence of the nephrotic syndrome. The majority of patients had their renal disease in the stage III-IV according K/DOQI classification (Table 1).

In this study, we have shown in patients with IgAN, that RANTES mRNA expression correlated significantly ($p<0.05$) with proteinuria (Fig. 1). Similarly, there were trends towards higher RANTES mRNA expression in patients with higher proteinuria who suffered from FSGS and MN, but they did not reach

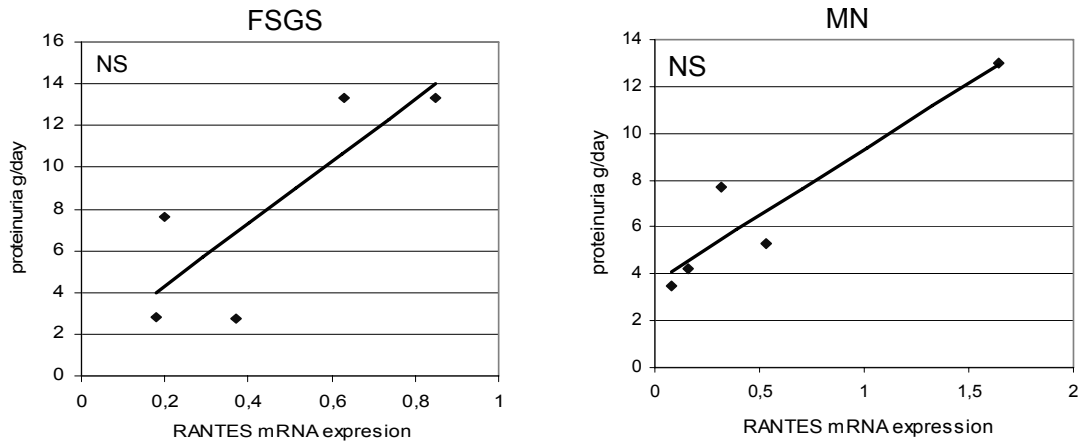

Fig. 2. Relationships between the RANTES mRNA intrarenal expression and the proteinuria level in kidneys with primary focal and segmental glomerulosclerosis (FSGS) and membranous nephropathy (MN).

statistical significance because of the small numbers of patients (Fig. 2). There was no clear relationship between MCP-1, TGF-β1, and TNF-α intrarenal mRNA expressions and proteinuria in patients with IgAN, FSGS and MN.

Furthermore, we did not observe significant differences in the pattern of intrarenal mRNA expression of RANTES, MCP-1, TNF-α and TGF-β1 among patients who suffered from IgAN, FSGS and MN (Fig. 3).

Discussion

We demonstrated a positive correlation between the intrarenal gene expression of the chemokine RANTES and proteinuria levels in patients with IgAN. Similarly, there were clear trends towards higher RANTES expression in patients with higher proteinuria who suffered from FSGS and MN. A large variety of injured tubular epithelial cells (Deckers *et al.* 1998), mesangial cells (Schlondorff *et al.* 1997), endothelium or stimulated fibroblasts (Rathanaswami *et al.* 1993) may secrete RANTES. The chemokine RANTES is thought to regulate interstitial inflammation by attracting lymphocytes and macrophages which secrete several profibrogenic regulators including TGF-β1, endothelin-1, angiotensin II and plasminogen activator inhibitor-1 (PAI-1) (Hirschberg and Wang 2005). These mediators may act on tubular cells as well as on fibroblasts. In our study, we have not observed any differences of cytokine gene expression among various glomerulonephritis cases. Therefore, there might be no specific correlation between the measured cytokine gene expression and the type of glomerular injury. RANTES may thus play an important role in the progression of glomerular diseases in case of the presence of clinically significant proteinuria.

Surprisingly, we did not observe any differences in MCP-1 gene expression. Mezzano *et al.* (2004) showed the MCP-1 protein is overexpressed within the tubuli of 11 patients diagnosed with diabetic nephropathy and proteinuria. Our study, however, analyzed the intrarenal MCP-1 gene expression. In this context, it would have been of interest to study MCP-1 also at the protein level. Unfortunately, there is not enough material left from the routine kidney biopsy to perform immunohistological staining. In another study, *in situ* hybridization revealed the overexpression of MCP-1, at both mRNA and protein levels, in 25 patients with MN (Mezzano *et al.* 2000). Thus, we cannot prove these observations in our study, despite the fact that our study included a larger number of subjects.

There were also no differences in either TNF-α or TGF-β1 mRNA expression. TNF-α has been observed frequently to be up-regulated during acute inflammatory disorders and also certain experimental therapy in nephrology uses anti-TNF monoclonal antibodies (Zaenker *et al.* 2004). On the contrary, TGF-β1 has been accepted to be associated with fibrogenesis. It seems that there are no relationships between both proinflammatory TNF-α and profibrogenic TGF-β1 and proteinuria. Repeated biopsies in the future may elucidate a role of these cytokines in the progression of the disease. Since we had no possibilities to obtain renal biopsy in healthy subjects, we are not able to speculate on the level of cytokine mRNA expression in our patients compared to the normal situation. TGF-β1, TNF-α, MCP-1 and RANTES are produced by all types of mononuclear cells, various cell types within the kidney or infiltrating cells.

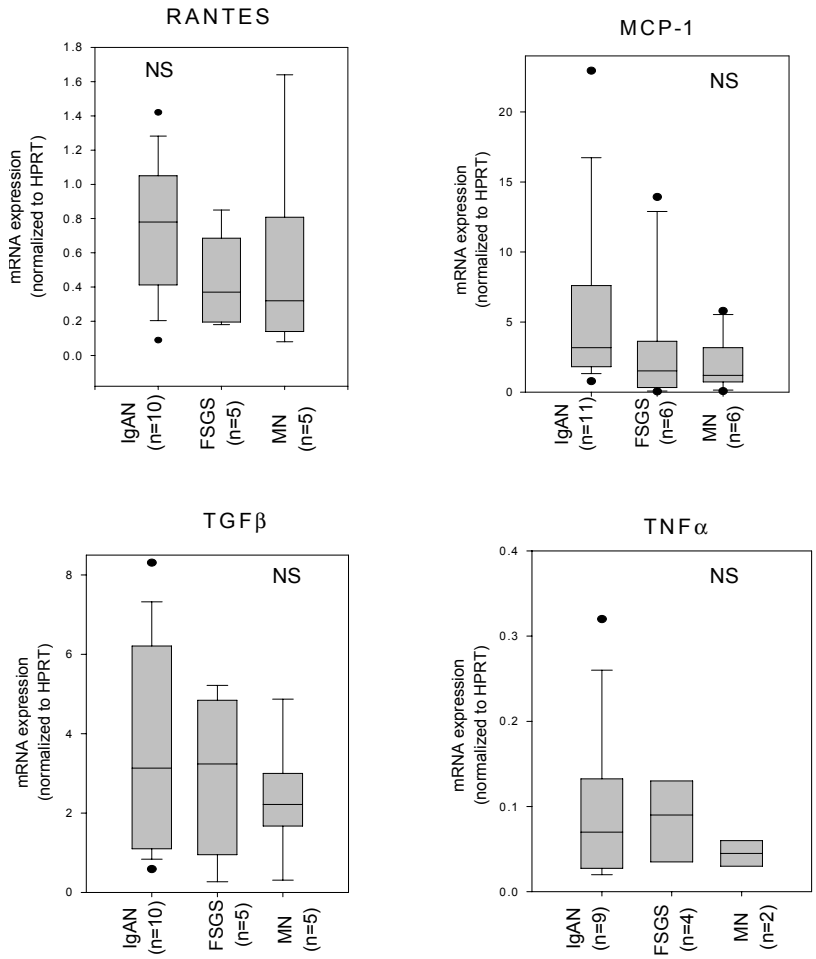

Fig. 3. Quantitative analysis of intrarenal expression of MCP-1, RANTES, TGF-β1 and TNF-α mRNA in patients with IgA nephropathy (IgAN), primary focal and segmental glomerulosclerosis (FSGS) and membranous nephropathy (MN). Expression data are normalized to the housekeeping gene HPRT ($2^{-\Delta Ct}$ method). NS: not significant; n: number of patients.

Some studies shows relationships between cytokine expression in peripheral blood mononuclear cells and kidney damage (Yano *et al.* 1997). Evaluation of cytokine/chemokine mRNA in the urine may provide additional information, however, renal biopsies remain best material reflecting particular changes in the kidney.

Surprisingly, despite small number of patients, our study is one of the largest hitherto published (Segerer *et al.* 2000, Kim *et al.* 2001, Lim *et al.* 2001, 2003). Since biopsy specimens were obtained for just routine morphology, there is not enough material for other immunohistological staining to detect cytokine protein expression within the renal tissue. Similarly, quantitative analysis of infiltrated mononuclear cells may be of interest to draw more definite conclusions. Prospective, costly, and multicenter trials with repeated biopsies may better elucidate the role of the above mentioned cytokines in glomerular proteinuric diseases. Finally, based on our results, it is likely that lymphocytes are involved in the renal injury as a response to proteinuria independently of the nature of renal diseases in man.

Acknowledgements

We thank J. Skibová for excellent statistical analysis, O. Remeš, M.D. for his help with data collection and T. Dear for English corrections. This work was supported by grant from Ministry of Health, Czech Republic NR/8913-4/2006.

References

DECKERS JG, VAN DER WOUDE FJ, VAN DER KOOIJ SW, DAHA MR: Synergistic effect of IL-1alpha, IFN-gamma, and TNF-alpha on RANTES production by human renal tubular epithelial cells in vitro. *J Am Soc Nephrol* **9**: 194-202, 1998.

HIRSCHBERG R, WANG S: Proteinuria and growth factors in the development of tubulointerstitial injury and scarring in kidney disease. *Curr Opin Nephrol Hypertens* **14**: 43-52, 2005.

IKEZUMI Y, KANNO K, KARASAWA T, HAN GD, ITO Y, KOIKE H, TOYABE S, UCHIYAMA M, SHIMIZU F, KAWACHI H: The role of lymphocytes in the experimental progressive glomerulonephritis. *Kidney Int* **66**: 1036-1048, 2004.

KIM YS, ZHENG S, YANG SH, KIM HL, LIM CS, CHAE DW, CHUN R, LEE JS, KIM S: Differential expression of various cytokine and chemokine genes between proliferative and non-proliferative glomerulonephritides. *Clin Nephrol* **56**: 199-206, 2001.

LIM CS, ZHENG S, KIM YS, AHN C, HAN JS, KIM S, LEE JS, CHAE DW, KOO JR, CHUN RW, NOH JW: Th1/Th2 predominance and proinflammatory cytokines determine the clinicopathological severity of IgA nephropathy. *Nephrol Dial Transplant* **16**: 269-275, 2001.

LIM CS, YOON HJ, KIM YS, AHN C, HAN JS, KIM S, LEE JS, LEE HS, CHAE DW: Clinicopathological correlation of intrarenal cytokines and chemokines in IgA nephropathy. *Nephrology (Carlton)* **8**: 21-27, 2003.

MEZZANO SA, DROGUETT MA, BURGOS ME, ARDILES LG, AROS CA, CAORSI I, EGIDO J: Overexpression of chemokines, fibrogenic cytokines, and myofibroblasts in human membranous nephropathy. *Kidney Int* **57**: 147-158, 2000.

MEZZANO SA, BARRIA M, DROGUETT MA, BURGOS ME, ARDILES LG, FLORES C, EGIDO J: Tubular NF-κB and AP-1 activation in human proteinuric renal disease. *Kidney Int* **60**: 1366-1377, 2001.

MEZZANO S, AROS C, DROGUETT A, BURGOS ME, ARDILES L, FLORES C, SCHNEIDER H, RUIZ-ORTEGA M, EGIDO J: NF-κB activation and overexpression of regulated genes in human diabetic nephropathy. *Nephrol Dial Transplant* **19**: 2505-2512, 2004.

OOTAKA T, SAITO T, YUSA A, MUNAKETA T, SOMA J, ABE K: Contribution of cellular infiltration to the progression of IgA nephropathy: a longitudinal immunocytochemical study on repeated biopsy specimens. *Nephrology* **1**: 135-142, 1995.

PLATZER C, ODE-HAKIM S, REINKE P, DOCKE WD, EWERT R, VOLK HD: Quantitative PCR analysis of cytokine transcription patterns in peripheral mononuclear cells after anti-CD3 rejection therapy using two novel multispecific competitor fragments. *Transplantation* **27**: **58**: 264-268, 1994.

RATHANASWAMI P, HACHICHA M, SADICK M, SCHALL TJ, McCOLL SR: Expression of the cytokine RANTES in human rheumatoid synovial fibroblasts. Differential regulation of RANTES and interleukin-8 genes by inflammatory cytokines. *J Biol Chem* **268**: 5834-5839, 1993.

REMUZZI G, BERTANI T: Pathophysiology of progressive nephropathies. *N Engl J Med* **339**: 1448-1456, 1998.

SEGERER S, CUI Y, HUDKINS KL, GOODPASTER T, EITNER F, MACK M, SCHLONDORFF D, ALPERS CE: Expression of the chemokine monocyte chemoattractant protein-1 and its receptor chemokine receptor 2 in human crescentic glomerulonephritis. *J Am Soc Nephrol* **11**: 2231-2242, 2000.

SCHLONDORFF D: The role of chemokines in the initiation and progression of renal disease. *Kidney Int* Suppl 49: S44-S47, 1995.

SCHLONDORFF D, NELSON PJ, LUCKOW B, BANAS B: Chemokines and renal disease. *Kidney Int* **51**: 610-621, 1997.

WATANABE T, KAWACHI H, IKEZUMI Y, YANAGIHARA T, ODA Y, SHIMIZU F: Glomerular CD8+ cells predict progression of IgA nephropathy. *Pediatr Nephrol* **16**: 561-567, 2001.

YANO N, ENDOH M, NOMOTO Y, SAKAI H, FADDEN K, RIFAI A: Phenotypic characterization of cytokine expression in patients with IgA nephropathy. *J Clin Immunol* **17**: 396-403, 1997.

ZAENKER M, ARBACH O, HELMCHEN U, GLORIUS P, LUDEWIG S, BRAASCH E: Crescentic glomerulonephritis associated with myeloperoxidase-antineutrophil-cytoplasmic antibodies: first report on the efficacy of primary anti-TNF-alpha treatment. *Int J Tissue React* **26**: 85-92, 2004.

Corresponding author

O. Viklický, Department of Nephrology, Transplant Laboratory, Institute for Clinical and Experimental Medicine, Videnska 1958, 140 00 Prague 4, Czech Republic. E-mail: ondrej.viklicky@medicon.cz

Original Article

NDT Nephrology Dialysis Transplantation

Association of advanced vasculopathy and transforming growth factor-beta1 gene expression with immunoglobulin A nephropathy progression

Irena Brabcova[1], Vladimir Tesar[5], Eva Honsova[3], Alena Lodererova[3], Eva Novotna[1], Dita Maixnerova[5], Miroslav Merta[5], Marcela Bürgelova[2], Petra Hribova[1], Jelena Skibova[4], Josef Zadražil[6], Jan Maly[2] and Ondrej Viklicky[1,2]

[1]Transplant Laboratory, Institute for Clinical and Experimental Medicine, Prague, Czech Republic, [2]Department of Nephrology, Institute for Clinical and Experimental Medicine, Prague, Czech Republic, [3]Department of Clinical and Transplant Pathology, Institute for Clinical and Experimental Medicine, Prague, Czech Republic, [4]Department of Statistics, Institute for Clinical and Experimental Medicine, Prague, Czech Republic, [5]Department of Nephrology, General Teaching Hospital, 1st Medical Faculty, Charles University Prague, Prague, Czech Republic and [6]3rd Internal Department, Teaching Hospital, Palacky University Olomouc, Olomouc, Czech Republic

Correspondence and offprint requests to: Ondrej Viklicky; E-mail: ondrej.viklicky@medicon.cz

Abstract

Background. The mechanism of IgA nephropathy (IgAN) progression remains ill-defined. In this prospective study, the prognostic role of clinical, histological and molecular markers over a 2-year follow-up was evaluated.

Methods. Fifty-one patients with biopsy-proven IgAN were followed for 24 months. Besides routine histology, the intrarenal gene expressions of cytokines and chemokines were quantified by reverse transcription quantitative real-time polymerase chain reaction, and the presence of lymphocytes and macrophages were immunohistochemically examined.

Results. Higher transforming growth factor-β1 and severe chronic vasculopathy (but not glomerulosclerosis, interstitial fibrosis or lymphocyte infiltrate) were associated with the IgAN progression 24 months after biopsy. The gene expression of chemokine (C–C motif) ligands 2 and 5, hepatocyte growth factor, bone morphogenic protein-7 and transforming growth factor-β1 and the interstitial infiltrate of T and B lymphocytes and macrophages were significantly associated with serum creatinine and glomerular filtration rate at the time of biopsy. The intrarenal chemokine (C–C motif) ligand 2 and hepatocyte growth factor gene expression were associated with the proteinuria.

Conclusions. Besides the known risk factors for chronic kidney disease, advanced vasculopathy and molecular signatures of fibrogenesis were associated with the IgAN progression.

Keywords: cytokines; gene expression; IgA nephropathy; interstitial infiltrate; vasculopathy

Introduction

Immunoglobulin A nephropathy (IgAN), mesangioproliferative glomerulonefritis, represents the most frequent histological finding in biopsies performed in the Western world and Asia. Since about 25% of patients develop end-stage renal disease, therapy for IgAN consumes significant economical resources. The pathogenesis of IgAN remains poorly understood, and it has been hypothesized that the mesangial depositions of immunoglobulin A are the result of autoimmune insult and antigen-independent altered glycosylation of immunoglobulin A [1]. The IgAN flare and immunologic activity is often difficult to estimate since the disease may last for decades before clinical manifestation. Predictors for poor outcome include both clinical and histological factors—all of which may be better markers of irreversible damage than the activity of the disease. The presence of renal dysfunction, proteinuria and hypertension at the time when the diagnosis is made has been considered to be associated with the disease progression and correlated with interstitial fibrosis as well [2]. However, the accuracy of these clinical markers is not sufficient to predict the risk for IgAN progression reliably or to guide more specific therapy for those in higher risk.

Recently, it has been hypothesized that molecular phenotypes of various diseases with broad forms of the manifestation, such as IgAN, might allow discriminating patients at risk for disease progression and personalizing their treatment [3–6]. Whole genome transcripts in micro-array analysis may prove molecular heterogeneity of

chronic kidney diseases (CKD) [7], but micro-array analyses are expensive, time consuming and often evaluated with improper statistics. Thus, gene expression analysis using the reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) method for the prediction of disease progression has recently been preferred with the aim of its use in clinical practice in the near future [8].

Interstitial fibrosis and tubular atrophy are universal consequences of renal damage of various aetiologies. In the experimental models of fibrogenesis, interstitial infiltration of mononuclear cells and up-regulation of various cytokines were observed. Transforming growth factor-β1 (TGF-β1) is known to be one of the most important factors in promoting mesangial matrix expansion and is suggested to be a key component of fibrogenesis cascade [9,10]. Although several reports suggest an antifibrogenic effect of hepatocyte growth factor (HGF), an increased deposition of matrix induced by HGF has also been reported. A proliferative state of target renal cells may influence the effects of HGF on extracellular matrix turnover
[11]. Contrarily, bone morphogenic protein-7 (BMP7) is a morphogen that is important for kidney development and has various antifibrotic properties [12]. The up-regulation of certain pro-inflammatory and profibrogenic genes such as chemokine (C–C motif) ligand 2 (CCL2, known also as MCP-1) or 5 (CCL5, known also as RANTES) has been shown to be a marker of progressive renal disease in humans
[13].

Similarly, the role of immune cell infiltration has also been hypothesized in the progression of CKD including IgAN. Immunohistochemical evaluation of tubulointerstitial inflammation was shown in a retrospective study to be a useful tool in determining the prognosis in IgAN and CD3 positive cell infiltration and strongly correlates with interstitial fibrosis and progression of IgAN [14].

The aim of this prospective multi-centre study was to evaluate the prognostic role of both the molecular phenotype of IgAN and the immune cell infiltration in the disease progression in a well-defined patient cohort.

Materials and methods

Patients

Fifty-one patients in whom primary IgAN was histologically diagnosed in three nephrology centres between January 2005 and May 2007 were prospectively enrolled in the study and followed for 24 months. Patients with Henoch–Schönlein purpura (HSP) systemic lupus erythematosus or with chronic liver diseases were not included in the study. There were 68.6% males; the median age at the time of biopsy was 39.1 [32.1–52.5] years; the median body mass index (BMI) was 26.8 [23.7–32.2] kg/m^2. The median serum creatinine was 25.8 [89.3–222.7] μmol/L, and the median glomerular filtration estimated by Modification of Diet in Renal Disease formula was 1.1 [0.5–1.7] mL/s. The median proteinuria at the biopsy time was 2.0 [0.9–4.5] g/day, and 83.7% of patients had significant erythrocyturia. The median serum IgA level was 3.3 [2.2–4.1] g/L. Parameters are expressed as median [25th–75th percentile]. Angiotensin-converting enzyme inhibitors (ACEi)/angiotensin II receptor blocker (ARB) therapy was used in 62.5% patients at the biopsy and in 90% and 89% of progressors and non-progressors, respectively, during the follow-up. None of our cohort had received immunosuppression before kidney biopsy.

All patients gave their written informed consent to participate in the study, and the Ethics Committee of the Institute for Clinical and Experimental Medicine in Prague approved the study protocol.

Renal biopsy

All biopsies were performed using a 14-gauge Tru-Cut needle (Uni-Cut Nadeln, Angiomed, Germany) guided by ultrasound (Toshiba, Power Vision 6000, Japan). Small portions (~2 mm) of renal tissue from the cortical or juxtamedullary zone were immediately stored in preserve solution (RNA later, Qiagen) for expression analysis, while the majority of renal tissue taken by core biopsy was used for routine histology performed by the standard method. Samples were routinely stained with haematoxylin and eosin, periodic acid–Schiff (PAS), aldehyde–fuchsin orange G (AFOG), Sirius red with elastic stain and periodic acid silver-methenamine (PASM). Histological examination was performed and revisited according to recent classification of IgAN [23].

Immunohistochemistry

Immunohistochemistry was performed on 4-μm-thick paraffin sections. The slides were deparaffinized in xylene and rehydrated in graded enthanols. After deparaffinization and rehydration, the slides were heated in a microwave oven for target retrieval. Endogenous peroxidase was blocked by 0.3% H_2O_2 in 70% methanol for 30 min.

Mononuclear cells

The slides were incubated with the primary antibody (CD3 polyclonal antibody from DAKO, Denmark; CD4 and CD8 monoclonal antibody from VECTOR laboratories, Burlingame, CA; CD20 and CD68 monoclonal antibodies from DAKO, Denmark), and detection of monoclonal antibodies was performed using Histofine Simple Stain MAX PO (Nichirei, Japan). Finally, the specimens were stained with 3,3 diaminobenzidine (DAKO, Denmark) and were counterstained with Harris's haematoxylin and embedded in Entellan (both from Merck, Germany). The number of positive cells per 1 mm^2 was calculated using Olympus DP-SOFT (Software Imaging Systems, Münster, Germany).

Transforming growth factor β1

The tissues were preincubated with a 10% horse serum (Vector laboratories, Burlingame, CA) for 20 min. Primary antibody (anti TGF-β1, clone TB21, Abcam) was applied for 30 min, diluted 100×. Detection of monoclonal antibody was done using a biotinylated horse anti mouse IgG (H+L) (Vector laboratories, Burlingame, CA) diluted 200× for 30 min. Then specimens were incubated with R.T.U. Vectastain Elite ABC Reagent (Vector Laboratories, Burlingame, CA) for 30 min. Finally, specimens were stained with 3,3 diaminobenzidine (DAKO, Denmark) for 3 min and were counterstained with Harris's haematoxylin before they were embedded in Entellan (both from Merck, Germany).

RNA isolation and RT-qPCR

After renal tissue was homogenized and total RNA extracted using RNA Blue (Top-Bio s.r.o. Czech Republic), it was reversely transcribed into complementary DNA (cDNA), as described elsewhere [15], and then the cytokine expression profile in renal tissue was analysed. Messenger RNA of chemokines (CCL2, CCL5) and cytokines (TGF-β1, HGF, BMP7) was quantified by RT-qPCR (Applied Biosystems 7900HT Fast Real-Time PCR System) using commercial TaqMan fluorogenic probes. Specific gene expression was calculated relative to the housekeeping gene hypoxanthine-guanine-phosphoribosyl-transferase (HPRT) using a comparative threshold cycle method. The plates also contain the calibrator sample for relative quantification ($2^{-\Delta\Delta Ct}$ method). All investigated mRNAs were measured in duplicates for each sample. The samples were considered negative if the Ct values exceeded 40 cycles.

Statistical analysis

The relationship between clinical values and mRNA expression was assessed using Spearman's correlation coefficient. Differences in mRNA expression or clinical parameters between groups were analysed using the Mann–Whitney test. The area below the receiver operating characteristic (ROC) curve and multivariate stepwise logistic regression were performed to estimate the risk for disease progression related with different transcripts, clinical values and mononuclear cell infiltration. In case of non-Gaussian distribution of values, the logarithmic transformation was used. A P-value <0.05 was considered to be statistically significant.

Table 1. Association of interstitial infiltrate and gene expression with clinical parameters (Spearman's correlation)

	CD3	CD4	CD8	CD20	CD68	*CCL5*	*CCL2*	*HGF*	*BMP7*	*TGF-β1*
Age (year)	0.41	n.s.	n.s.	n.s.	0.36	0.45	0.42	0.34	n.s.	0.32
Creatinine (mg/dL)	0.74	n.s.	0.63	0.58	0.60	0.63	0.49	0.35	−0.52	0.29
GFR (mL/min)	−0.67	n.s.	−0.52	−0.51	−0.61	−0.57	−0.44	−0.32	0.40	−0.37
Proteinuria (g/day)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.37	0.41	n.s.	n.s.

$r > 0.279$, $P < 0.05$.
$r > 0.361$, $P < 0.01$.
$r > 0.451$, $P < 0.001$.
n.s., not significant.

Results

Cross-sectional study

To evaluate the association of intrarenal gene expression patterns or lymphocyte or macrophage infiltrate with clinical and histological parameters, several univariate analyses were performed.

Associations of gene expression patterns and mononuclear cell infiltration with the histological presence of crescents at the biopsy

Although the morphology in IgAN may vary, we did not find any significant differences between the expression profiles of analysed genes and the presence of crescents in the biopsy. Similarly, the infiltration of T-helper cells (CD4+), cytotoxic T cells (CD8+) and B cells (CD20+) in the interstitium and glomerular area were not associated with the presence of cellular crescents. Infiltration of T cells (CD3+) and macrophages (CD68+) cells in the interstitium were significantly higher in biopsy samples with cellular crescents ($P = 0.02$ and $P = 0.05$, respectively) (data are not shown).

Associations of mononuclear cell infiltration and gene expression patterns with renal function, proteinuria and haematuria at the biopsy

To determine the relationship between intrarenal cell infiltration, cytokine gene expression profiles and renal injury, the correlation between mononuclear cell infiltration and gene expression patterns with clinical parameters was analysed. The gene expression patterns of analysed molecules *CCL2*, *CCL5*, *HGF*, *BMP7* and *TGF-β1* were significantly related to glomerular filtration rate (GFR) and serum creatinine at the time of biopsy (Table 1). The interstitial infiltrate of T cells, cytotoxic T cells, B cells and macrophages (CD3+, CD8+, CD20+ and CD68+ cells) was significantly higher in patients with decreased renal function (Table 1). There was no relation between analysed parameters and T-helper cell infiltration (CD4+). Glomerular infiltrate was not associated with renal function.

Cytokine gene expressions and mononuclear cell infiltration were associated with proteinuria. Proteinuria significantly correlated with the intrarenal *CCL2* and *HGF* mRNA expression ($P < 0.01$, $r = 0.37$; $P < 0.01$, $r = 0.41$, respectively) and negatively correlated with macrophage infiltration in the glomeruli ($P < 0.05$, $r = 0.36$).

A gene transcript of *HGF*, a molecule with antifibrotic properties, was higher in patients without erythrocyturia ($P < 0.05$), and glomerular macrophage infiltration (CD68+) was significantly higher in patients with erythrocyturia ($P < 0.05$).

Association of interstitial infiltration with the gene expression patterns

The interstitial infiltration of CD3+, CD8+, CD20+ and CD68+ positive cells significantly correlated with the mRNA of target genes (Table 2). Mononuclear cell invasion in glomeruli was rare and not significant. There was only a positive correlation between *BMP7* mRNA expression and infiltrate of both CD3- and CD68-positive cells ($P < 0.05$, $r = 0.36$; $P < 0.01$, $r = 0.37$) (data are not shown).

Longitudinal study

Based on the disease progression during the 24-month follow-up after kidney biopsy, two patient subgroups were identified. Ten patients were classified as 'progressors', in whom serum creatinine at the biopsy increased for more than 25% of baseline values ($n = 4$) or who had renal failure during 2 years follow-up ($n = 6$). Patients in whom serum creatinine decreased during the 24-month follow-up or remained stable within normal range were classified as 'non-progressors' ($n = 37$). Four patients in whom the follow-up was not available were excluded from the analysis.

Univariate analysis

Patients in the 'progressor' group were older, had poorer GFR and higher serum creatinine at the biopsy. Morphological evaluation found higher chronic vascular changes

Table 2. Association of interstitial infiltrate with gene expression (Spearman's correlation)

Gene transcript	*CCL5*	*CCL2*	*HGF*	*BMP7*	*TGF-β1*
CD3+	0.61	0.37	n.s.	−0.43	0.32
CD4+	n.s.	n.s.	n.s.	n.s.	n.s.
CD8+	0.49	0.37	n.s.	−0.53	n.s.
CD20+	0.39	0.34	0.31	−0.49	n.s.
CD68+	0.43	0.42	n.s.	−0.47	n.s.

$r > 0.279$, $P < 0.05$.
$r > 0.361$, $P < 0.01$.
$r > 0.451$, $P < 0.001$.
n.s., not significant.

in the 'progressor' group (P < 0.02, relative risk (RR) = 3.7). Similarly, the higher intrarenal expression of TGF-β1 mRNA and CD68+ cell infiltrate was found in the 'progressor' group (Table 3). There were no differences in the histological presence of crescents, glomerulosclerosis, interstitial fibrosis, ACEi/ARB treatment or used immunosuppression (Table 3).

ROC analysis

For determination of the risk for IgAN progression in dependence of renal gene expression and mononuclear cell infiltration during the 24-month follow-up after kidney biopsy, the ROC analysis was designed, and the critical cut points were adjusted. A higher *TGF-β1*, *HGF* and *CCL5* intrarenal mRNA expression (Relative quantity (RQ) > 1.45, RQ > 1.05 and RQ > 31.9, respectively) and higher macrophage infiltration (cell number >59.7) were associated with the risk for disease progression (RR 7.1 for *TGF-β1*, 5.5 for *HGF*, 4.9 for *CCL5* and 7.5 for CD68, respectively) (P < 0.05) (Figure 1). The patterns of *CCL2* and *BMP-7* gene expression did not impact the disease progression (data are not shown). Despite trends towards frequent interstitial infiltration in the 'progressor' group, both univariate and ROC analyses found the interstitial infiltrate of T cells, T-helper cells, cytotoxic T cells and B cells to have no significant relationship with the disease progression (Table 3).

Multivariate analysis

The influence of multiple variables (age at diagnosis, GFR, serum creatinine, up-regulation of *HGF*, *TGF-β1* and

Table 3. Demographical, clinical, cellular and molecular parameters in patients with and without 24 months disease progression

Clinical variables	'Progressors'	'Non-progressors'	P
N	10	37	
Male (%)	70.0	78.4	0.68
Age (years)	55.6 [26.1–65.6]	38.9 [19.3–64.6]	0.05
BMI (kg/m^2)	33.0 [20.9–36.8]	24.3 [19.1–40.4]	0.12
Creatinine (μmol/L)	309.8 [64.0–534.8]	108.4 [63.0–590.0]	0.02
GFR (mL/s)	0.4 [0.1–1.3]	1.2 [0.2–5.0]	0.00
Proteinuria (g/day)	4.2 [0.4–7.8]	2.1 [0.1–11.0]	0.19
Erythrocyturia (%)	88.9	83.3	1.00
Serum IgA (g/L)	4.4 [1.6–8.0]	3.3 [1.1–5.52]	0.21
Systolic blood pressure (mmHg)	153 [130–210]	140 [90–220]	0.07
Diastolic blood pressure (mmHg)	90 [80–120]	90 [60–130]	0.80
Cholesterol (mmol/L)	4.9 [2.9–8.0]	5.5 [2.7–10.5]	0.32
Triglycerides (mmol/L)	1.5 [0.6–3.9]	2.0 [0.4–6.1]	0.58
Uric acid (μmol/L)	419.0 [87.0–482.0]	398.0 [190.0–675.0]	0.90
Immunosuppression (%)[a]	60.0	29.7	0.19
Prednisone alone	50.0	61.5	1.00
Prednisone + cyclophosphamide	50.0	38.5	
Gene expression (RQ)			
CCL5	46.0 [13.5–122.5]	19.4 [0.7–72.9]	0.06
CCL2	0.3 [0.1–0.8]	0.2 [0.0–0.7]	0.29
HGF	1.8 [1.0–3.4]	1.2 [0.1–6.1]	0.07
BMP7	0.9 [0.3–2.5]	0.9 [0.4–7.6]	0.45
TGF- β1	1.7 [0.7–5.1]	1.2 [0.0–4.9]	0.01
Interstitial infiltration			
CD3+ (cells/mm^2)	521.9 [67.2–881.9]	182.9 [20.0–1265.3]	0.07
CD4+ (cells/mm^2)	61.4 [0.0–201.9]	30.5 [0.0–224.6]	0.99
CD8+ (cells/mm^2)	124.5 [29.0–509.6]	88.1 [6.1–613.8]	0.11
CD20+ (cells/mm^2)	59.6 [3.9–292.7]	13.9 [0.0–265.6]	0.13
CD68+ (cells/mm^2)	68.7 [15.3–143.9]	23.9 [0.0–105.7]	0.02
Histology			
Sclerotic glomeruli (%)	41.7 [0.0–66.7]	11.4 [0.0–64.3]	0.08
Segmental sclerosis (%)	46.4 [4.0–100.0]	32.1 [0.0–100.0]	0.29
Mesangial hypercellularity >6 cells (%)	11.1	39.4	0.23
Endocapillary hypercellularity (%)	62.5	54.5	1.00
Cellular crescents (%)	70.0	45.5	0.29
IF/TA ≥2 (%)	70.0	39.4	0.15
ti ≥2 (%)	70.0	45.5	0.28
cv ≥2 (%)	60.0	17.9	0.02
ah ≥ 2 (%)	70.0	42.4	0.16

Parameters are expressed as median [minimum − maximum] or in percentage.

[a]Follow-up data. Histology classification was performed according to The Oxford Classification of IgA nephropathy: pathology definitions, correlations and reproducibility [23].

Interstitial fibrosis/tubular atrophy (IF/TA), tubulointerstitial infiltrate (ti), chronic vasculopathy (cv) and arteriolar hyalinosis (ah) scoring systems were adopted from Banff classification [8].

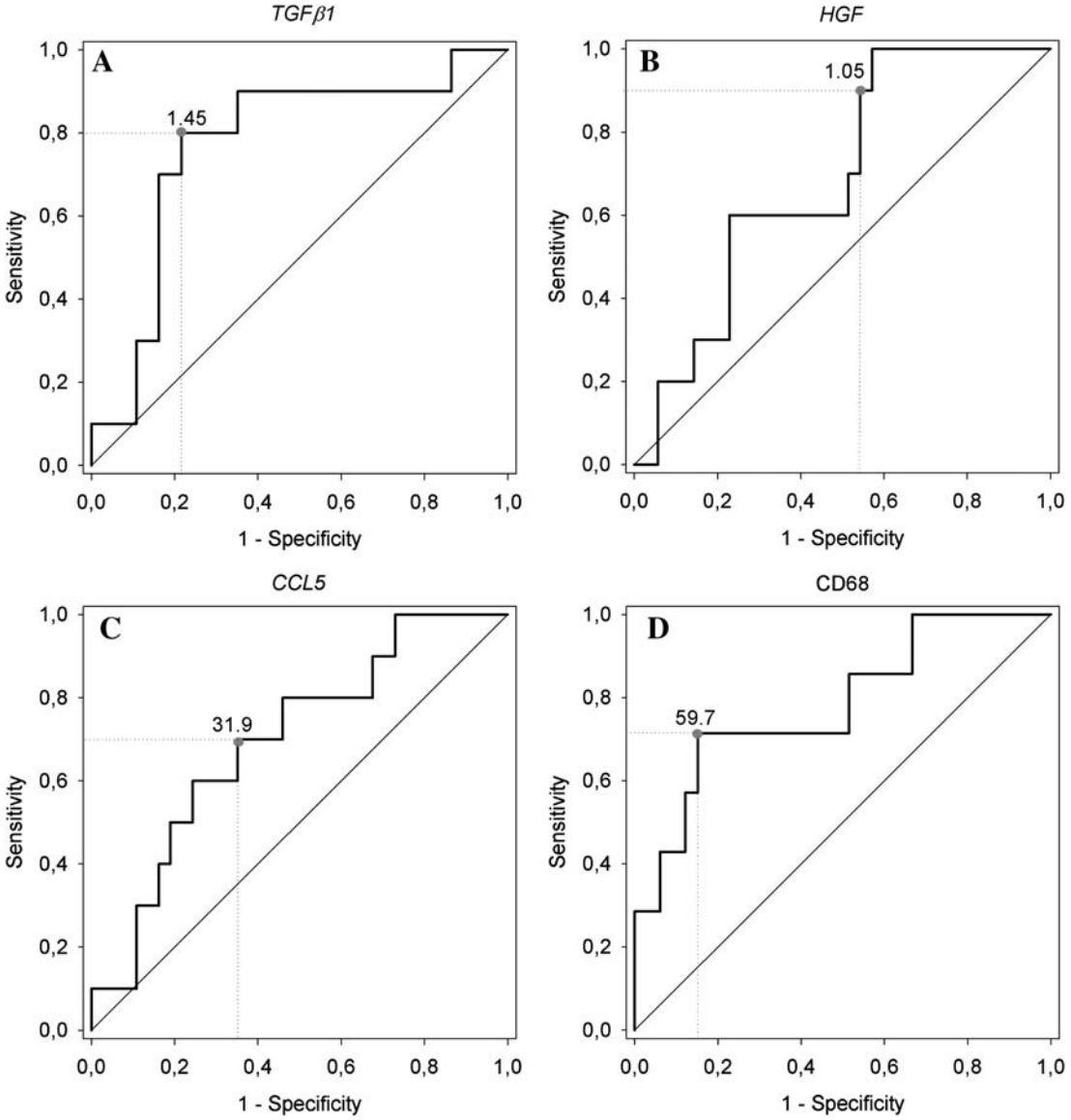

Fig. 1. Receiver operating characteristic (ROC) curves for IgAN progression according to the *TGF-β1*, *HGF*, *CCL5* gene expression and CD68+ cell infiltration. Up-regulation of these markers significantly determined the progression of IgAN at 2 years after diagnosis. (**A**) *TGF-β1* (cut point = 1.45, sensitivity = 80.0%, specificity = 78.4%, area under the curve (AUC) = 0.77, SE = 0.09). (**B**) *HGF* (cut point = 1.05, sensitivity = 90.0%, specificity = 45.7%, AUC = 0.69, SE = 0.08). (**C**) CCL5 (cut point = 31.9, sensitivity = 70.0%, specificity = 64.9%, AUC = 0.70, SE = 0.09). (**D**) CD68 (cut point = 59.7, sensitivity = 71.4%, specificity = 84.8%, AUC = 0.78, SE = 0.11).

CCL5, chronic vasculopathy score and immunosupression) on IgAN progression from univariate analysis was analysed by multivariate regression model. CD68+ cells were excluded from this model because of missing data from three progressor patients (there was not enough material for cell account). This model showed advanced chronic vasculopathy (OR = 7.1) and higher *TGF-β1* expression (OR = 12.2) to be associated with 2-year disease progression (Table 4).

Association of TGF-β1 and chronic vasculopathy

There was no statistically significant association between *TGF-β1* mRNA expression and chronic vasculopathy score (P = 0.27) at the time of biopsy. TGF-β1 immuno-

Table 4. Markers of IgAN progression in multivariate analysis

		Multivariate logistic regression			
	Univariate P	Coefficient	OR	95%C.I.	P
Age (year)	0.05	–	–	–	n.s.
Creatinine (μmol/L)	0.02	–	–	–	n.s.
GFR (mL/s)	0.00	–	–	–	n.s.
Immunosuppression (%)	0.19	–	–	–	n.s.
CCL5 > 31.9	0.06	–	–	–	n.s.
HGF > 1.05	0.07	–	–	–	n.s.
TGF-β1 > 1.45	0.01	2.5	12.2	1.6–91.0	0.007
cv ≥ 2	0.02	2.0	7.1	1.0–50.2	0.03

n.s., P > 0.10.

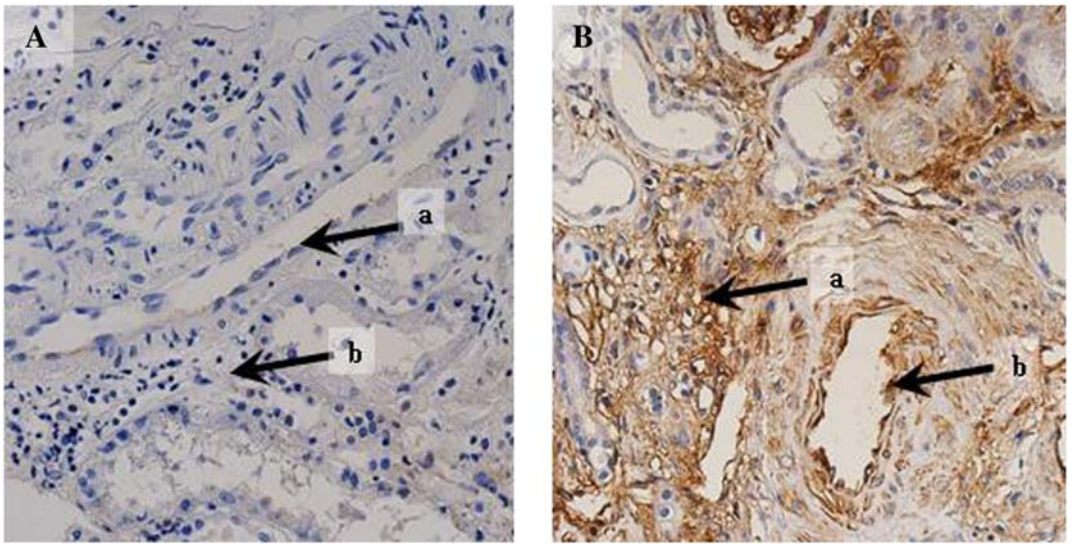

Fig. 2. (**A**) Negative TGF-β1 immunohistochemistry in patients without 24 months IgAN progression; a, the arterial wall; b, the interstitium. (**B**) Positive TGF-β1 imunohistochemistry in progressors; a, focal positivity in the interstitium with inflammation and fibrosis; b, positivity within the thickened intima of the smooth muscle artery.

histochemical staining revealed the focal positivity both within the inflammatory-cell-rich interstitium and within the thickened smooth muscle artery intima in patients in whom IgAN progressed (Figure 2).

Discussion

The IgAN is characterized by an extreme variability in clinical course and by the unpredictability of the ultimate outcome. The different outcome of IgAN with similar clinical and morphological manifestations suggests its molecular heterogeneity. In this prospective study, we hypothesized that the expression patterns of several genes with the known function in fibrosis regulation along with immune cells infiltration were associated with the disease progression. At the IgAN diagnosis, we found both several gene transcripts and immune cell infiltrate to correlate with the known risk factors of disease progression such as poor renal function and proteinuria. However, in the 24-month follow-up, besides clinical parameters, the IgAN progression was associated only with the chronic vasculopathy and TGF-β1 gene expression.

Katafuchi *et al*. [16] evaluated the role of vascular disease in the progression of IgAN by morphometric analysis in 71 patients. Interestingly, vessel area and hypertension were equally important as predictors of glomerular sclerosis and IgAN progression. Similarly, recent data from a Japan study [17] showed that histological grade involving also vasculopathy at initial biopsy was considered to be a reliable parameter to predict IgAN progression among prospectively followed 2283 patients.

Chronic vasculopathy, the consequence of hypertension and ageing, causes renal ischaemia. As a result of renal ischaemia, angiotensin II-dependent TGF-β1 overexpression was identified to be a critical component of renal fibrogenesis [18]. TGF-β1 is a central stimulus of the events leading to chronic progressive kidney disease, having been implicated in the regulation of cell proliferation, hypertrophy, apoptosis and fibrogenesis. By multiple mechanisms, this growth factor acts as a major regulator of extracellular matrix production and degradation; it stimulates synthesis of extracellular matrix (collagen, fibronectin and proteoglycans), enhances expression of integrins and reduces activities of matrix-degrading proteases [19]. Recently, novel mRNA and protein signatures related also with TGF-β1 signalling were shown to be predictive for CKD progression in both mice and humans [20]. This observation is in line with the results of our study.

Interstitial inflammation is a prominent feature associated with the severity of renal injury and progressive kidney failure. In the cross-sectional part of the study, we showed interstitial infiltrate of T and B lymphocytes along with macrophages to correlate with the up-regulation of chemokine genes that attract lymphocytes and monocytes into the site of injury. These cells are thought to actively participate in fibrogenesis [19]. Proteinuria is widely accepted as a risk for CKD progression [21]; here, we showed an association of the proteinuria with chemokine and growth factor gene expression. Contrary to this cross-sectional data, only macrophage interstitial infiltration but not lymphocyte infiltrate was shown to predict the IgAN progression in the longitudinal part of the study. This contrasts with retrospective studies where tubulointerstitial inflammation determined IgAN prognosis [14,22]. These studies provided, however, no relevant information regarding the used immunosuppression, while in our study, both 'progressor' and 'non-progressor' groups did not statistically differ in immunosuppressive therapy. This may reflect the presence of active disease phenotype with dense mononuclear infiltrates at the early stage of the disease that was successfully converted. We also did not prove the prognostic role of interstitial fibrosis or glomerulosclerosis. The meta-analysis of 30 studies that, besides clinical factors, also evaluated prognostic value of morphological lesions was inconsistent because histology was characterized either by overall score or by semiquantitative grading of individual

glomerular, tubular, interstitial and vascular changes. When single lesions were analysed separately, glomerulosclerosis and interstitial fibrosis appeared to be the predictors of IgAN progression [2].

Besides known risk factors for CKD progression such as higher proteinuria and poor renal function, in our study we have shown advanced vasculopathy and molecular signatures of fibrogenesis to be associated with the short-term IgAN progression. Taking into account also higher patients' age, we assume that the clinical diagnosis of the disease in the 'progression' group was made in the longer interval after its first flare. For adapting treatment decisions to IgAN molecular phenotype in the light of our limited sample size, larger prospective multi-centre and molecular marker-based clinical trials are warranted to validate these results.

Acknowledgement. This work was supported by Internal Grant Agency from the Ministry of Health No.:NR/8913-4/2006. A part of the study was presented in the World Congress of Nephrology, 22–26 May 2009, Milan, Italy. The authors are indebted to the patients and nurses for cooperation and help.

Conflict of interest statement. None of the authors have a financial interest in a business or commercial entity, or an intellectual property interest, that relates to the manuscript.

References

1. Julian BA, Novak J. IgA nephropathy: an update. *Curr Opin Nephrol Hypertens* 2004; 13: 171–179
2. D'Amico G. Natural history of idiopathic IgA nephropathy: role of clinical and histological prognostic factors. *Am J Kidney Dis* 2000; 36: 227–237
3. Viklicky O, Hribova P, Volk HD *et al*. Molecular phenotypes of acute rejection predict renal graft prognosis. *J Am Soc Nephrol* 2010; 21: 173–180
4. Itadani H, Mizuarai S, Kotani H. Can systems biology understand pathway activation? Gene expression signatures as surrogate markers for understanding the complexity of pathway activation. *Curr Genomics* 2008; 9: 349–360
5. Schmid H, Henger A, Kretzler M. Molecular approaches to chronic kidney disease. *Curr Opin Nephrol Hypertens* 2006; 15: 123–129
6. Rovin BH, McKinley AM, Birmingham DJ. Can we personalize treatment for kidney diseases? *Clin J Am Soc Nephrol* 2009; 4: 1670–1676
7. Schwab K, Witte DP, Aronow BJ *et al*. Microarray analysis of focal segmental glomerulosclerosis. *Am J Nephrol* 2004; 24: 438–447
8. Solez K, Colvin RB, Racusen LC *et al*. Banff 07 classification of renal allograft pathology: updates and future directions. *Am J Transplant* 2008; 8: 753–760
9. Isaka Y, Fujiwara Y, Ueda N *et al*. Glomerulosclerosis induced by in vivo transfection of transforming growth factor-beta or platelet-derived growth factor gene into the rat kidney. *J Clin Invest* 1993; 92: 2597–2601
10. Schnaper HW, Jandeska S, Runyan CE *et al*. TGF-beta signal transduction in chronic kidney disease. *Front Biosci* 2009; 14: 2448–2465
11. Esposito C, Parrilla B, Cornacchia F *et al*. The antifibrogenic effect of hepatocyte growth factor (HGF) on renal tubular (HK-2) cells is dependent on cell growth. *Growth Factors* 2009; 27: 137–180
12. Sugimoto H, Grahovac G, Zeisberg M *et al*. Renal fibrosis and glomerulosclerosis in a new mouse model of diabetic nephropathy and its regression by bone morphogenic protein-7 and advanced glycation end product inhibitors. *Diabetes* 2007; 56: 1825–1833
13. Mezzano SA, Barria M, Droguett MA *et al*. Tubular NF-kappaB and AP-1 activation in human proteinuric renal disease. *Kidney Int* 2001; 60: 1366–1377
14. Myllymaki JM, Honkanen TT, Syrjanen JT *et al*. Severity of tubulointerstitial inflammation and prognosis in immunoglobulin A nephropathy. *Kidney Int* 2007; 71: 343–348
15. Platzer C, Ode-Hakim S, Reinke P *et al*. Quantitative PCR analysis of cytokine transcription patterns in peripheral mononuclear cells after anti-CD3 rejection therapy using two novel multispecific competitor fragments. *Transplantation* 1994; 58: 264–268
16. Katafuchi R, Vamvakas E, Neelakantappa K *et al*. Microvascular disease and the progression of IgA nephropathy. *Am J Kidney Dis* 1990; 15: 72–79
17. Goto M, Wakai K, Kawamura T *et al*. A scoring system to predict renal outcome in IgA nephropathy: a nationwide 10-year prospective cohort study. *Nephrol Dial Transplant* 2009; 24: 3068–3074
18. Border WA, Noble NA. Interactions of transforming growth factor-beta and angiotensin II in renal fibrosis. *Hypertension* 1998; 31: 181–188
19. Wynn TA. Cellular and molecular mechanisms of fibrosis. *J Pathol* 2008; 214: 199–210
20. Ju W, Eichinger F, Bitzer M *et al*. Renal gene and protein expression signatures for prediction of kidney disease progression. *Am J Pathol* 2009; 174: 2073–2085
21. Jafar TH, Stark PC, Schmid CH *et al*. Progression of chronic kidney disease: the role of blood pressure control, proteinuria, and angiotensin-converting enzyme inhibition: a patient-level meta-analysis. *Ann Intern Med* 2003; 139: 244–252
22. Van Es LA, de Heer E, Vleming LJ *et al*. GMP-17-positive T-lymphocytes in renal tubules predict progression in early stages of IgA nephropathy. *Kidney Int* 2008; 73: 1426–1433
23. Roberts IS, Cook HT, Troyanov S *et al*. The Oxford classification of IgA nephropathy: pathology definitions, correlations, and reproducibility. *Kidney Int* 2009; 76: 546–556

Received for publication: 31.12.09; Accepted in revised form: 24.6.10

Kidney & Blood Pressure Research

Kidney Blood Press Res 2007;30:273–282
DOI: 10.1159/000105134

Received: January 25, 2007
Accepted: May 22, 2007
Published online: July 4, 2007

Intrarenal Cytokine and Chemokine Gene Expression and Kidney Graft Outcome

Petra Hribova[a] Jiri Lacha[a, b, †] Katja Kotsch[e] Hans-Dieter Volk[e]
Irena Brabcova[a] Jelena Skibova[c] Stefan Vitko[d] Ondrej Viklicky[a, b]

[a]Transplant Laboratory, [b]Department of Nephrology, [c]Department of Statistics, and [d]Transplant Center, Institute for Clinical and Experimental Medicine, Prague, Czech Republic; [e]Institute of Medical Immunology, Universitätsmedizin Charité Berlin, Campus Mitte, Berlin, Germany

Key Words
Kidney transplantation · Chronic allograft nephropathy · Cytokines · Chemokines · Gene expression · Proteinuria

Abstract
Aims: Proinflammatory cytokines are thought to play an important role in various kidney graft diseases resulting in interstitial fibrosis and tubular atrophy frequently found in case biopsies. To explore the role of various cytokines and chemokines in the long-term graft outcome, the transcription patterns of their genes in kidney allograft biopsies were evaluated. ***Methods:*** The real-time RT-PCR was used to identify intragraft mRNA expression of cytokines and chemokines in 74 kidney graft recipients and the results were correlated with histological and clinical parameters and long-term graft outcome. ***Results:*** We observed up-regulated IL-10 ($p < 0.001$), TGF-β_1, IL-6, MCP-1, RANTES ($p < 0.01$) and TNF-α ($p < 0.05$) mRNA expression in patients with chronic allograft nephropathy (CAN) as compared to controls. There were positive correlations between the mRNA expression of IL-6 ($p < 0.001$), IL-10 ($p < 0.01$), TNF-α, MCP-1 ($p < 0.05$) and the proteinuria. The up-regulation of intrarenal MCP-1 in patients with CAN increased the risk for the graft failure within the next 42 months (OR 5.1, $p < 0.05$). Kaplan-Meier survival analysis revealed that proteinuria and higher intragraft expression of TGF-β_1 and MCP-1 predict a poor kidney graft outcome. ***Conclusion:*** Expression patterns of intrarenal proinflammatory genes might discriminate patients at a higher risk for the earlier allograft failure.

The study was presented at the World Transplant Congress, Boston, Mass., 2006.

Introduction

Although the significant improvement in the 1-year survival rate of renal transplants and patients has been achieved in the last decades, the fate of renal allograft has not been substantially changed in the long term [1]. Both interstitial fibrosis and tubular atrophy as a result of various graft injuries are involved in chronic allograft nephropathy (CAN) [2–5]. CAN defined in the Banff 97 classification is frequently found in failing grafts. Alloantigen-dependent risk factors for the interstitial fibrosis/tubular atrophy (IF/TA) development have been shown to be acute and subclinical rejection and chronic humoral rejection, whereas alloantigen-independent factors such as poor donor organ quality, ischemia/reperfusion injury, infections, calcineurin inhibitor nephrotoxicity or hypertension may play a role at the same time

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

Accessible online at:
www.karger.com/kbr

Ondrej Viklicky, Assoc. Prof., MD, PhD
Department of Nephrology
Institute for Clinical and Experimental Medicine
Vídeňská 1958/9, CZ–140 21 Prague 4 (Czech Republic)
Tel. +420 2 6136 6070, Fax +420 2 6136 3117, E-Mail ondrej.viklicky@medicon.cz

Table 1. Patient demographic and clinical characteristics (mean ± SD values)

Patient group	Controls	CAN
Biopsies	30	44
Females	12 (40%)	13 (30%)
Patient age, years	43.4 ± 12.9	44.8 ± 12.3
Donor age, years	45.9 ± 14.3	40.9 ± 17.8
Living donor kidney transplants, %	3	7
HLA mismatches[1]	3.3 ± 1.4	3.6 ± 1.2
Panel-reactive antibodies	19.0 ± 27.3	19.7 ± 24.2
Post-transplant time, months	15.7 ± 10.4	44.8 ± 50.1**
Total follow-up, months	46.0 ± 35.2	130.6 ± 130.5**
History of acute rejection, %	28	50
Diabetes, %	17	16
Hypertension, %	93	98
Use of ACE inhibitors, %	37	59
Use of hypolipidemics, %	53	66
Glomerular filtration rate, ml/s	1.1 ± 0.4	0.8 ± 0.4*
Serum creatinine, μmol/l	149 ± 43	219 ± 103*
Proteinuria, g/l	0.39 ± 0.65	1.30 ± 2.17*
Serum cholesterol, mmol/l	5.6 ± 1.0	5.8 ± 1.3
Serum triglyceride, mmol/l	2.4 ± 1.4	2.9 ± 1.6

* $p < 0.01$; ** $p < 0.001$.
[1] Mismatches between donor and recipient in HLA A, B, and DR loci.

[6–9]. Infiltrating cells producing a broad spectrum of cytokines, chemokines and growth factors have been frequently found within the injured allografts [10–13]. Animal models of acute and chronic kidney allograft rejection showed an increased intragraft expression of TGF-β_1 [14], TNF-α, IFN-γ, RANTES, MCP-1 and IL-6 [15–17] compared to control animals without rejection. Chemokines are important activators and attractants of leukocytes infiltrating the graft. RANTES is expressed already in early stages after transplantation and MCP-1, especially in late stages after kidney transplantation [16, 18]. However, all these results obtained from animal models were only partially confirmed in human studies [11]. Recent knowledge shows different and inconsistent results of gene expressions in CAN according to the Banff 97 classification that involves IF/TA, vasculopathy and glomerulopathy [19–21]. To describe the potential of such risk factors in the CAN development, we evaluated intrarenal cytokine (TNF-α, IL-6, IL-10), chemokine (MCP-1, RANTES) and growth factor (TGF-β_1) mRNA expression in the large cohort of patients with biopsy-proven CAN or normal findings and correlated patterns of gene expression with morphology and kidney graft outcome.

Materials and Methods

Patients

For the purpose of this study, we used 74 biopsies that revealed either CAN according to the Banff 97 classification or normal morphological findings and in which a part of the biopsy specimens was used for mRNA isolation. Biopsies were performed between November 2001 and June 2003 because of late graft function deterioration (n = 47) or according to protocol (n = 27). There were 49 males and 25 females; the mean age at the time of biopsy was 47.0 ± 12.6 years. Patient demographic and clinical data are summarized in table 1. Cyclosporine A was the cornerstone immunosuppressant in 58 of them, tacrolimus in the rest. All patients were regularly followed. The renal function of patients was monitored up to 42 months after renal biopsy.

From the 430 biopsies performed in our center during this period, only those were included into the study that revealed either CAN or normal morphology and the sufficient amount of RNA for analyses was available. All patients gave their written informed consent to participate in the study and the Ethics Committee of the Institute for Clinical and Experimental Medicine in Prague approved the study protocol.

Renal Biopsies and Histomorphology

All biopsies were done by a 14-gauge Tru-Cut needle (Uni-Cut Nadeln, Angiomed, Germany) guided by ultrasound (Toshiba, Power Vision 6000, Japan). Small portions of renal tissue from the cortex or juxtamedullary zone were immediately frozen in liquid nitrogen and stored at –80°C for expression analysis, while most of the renal tissue taken by core biopsy was used for routine histology performed by the standard method. Tissues were fixed in 10% formalin for 15–30 min and then processed in a TPC-15 tissue processor (Medite Histotechnik, Germany). 4-μm-thick paraffin-embedded tissue sections were stained with hematoxylin and eosin, periodic acid-Schiff, aldehyde-fuchsin orange G, Sirius red with elastic stain and periodic acid-silver methenamine. Biopsy tissues were scored on the basis of the Banff 97 working classification [4]. 44 specimens with CAN were obtained (32 case and 12 protocol biopsies) and 30 specimens obtained from patient with stable and good renal function (n = 15) or with only transient deterioration with spontaneous improvement (n = 15), which showed a normal histological pattern of renal tissue served as controls. Banff 97 scores in patient groups are summarized in table 2.

Clinical Data

The following clinical variables were recorded in the patients: gender, age at the time of transplantation, donor age, the origin of kidney graft (deceased or living donor), the number of HLA mismatches, maximal panel-reactive antibodies, serum creatinine, GFR estimated using the Cockcroft-Gault formula [22], proteinuria, serum cholesterol and triglyceride levels, the occurrence of diabetes mellitus and hypertension, use of angiotensin-converting enzyme (ACE) inhibitors, use of hypolipidemics, the history of acute rejection and delayed graft function incidence (defined as a need of post-transplant dialysis).

Patients with CAN did not differ significantly from the control group in patient age, gender, donor age, the origin of kidney graft, number of HLA mismatches, maximal panel-reactive antibodies, serum cholesterol and triglyceride levels, the occurrence

of diabetes mellitus, the occurrence of hypertension, use of ACE inhibitors, use of hypolipidemics, the incidence of acute rejection and delayed graft function. Patients suffering from CAN revealed serum creatinine level and proteinuria to be higher and GFR to be lower ($p < 0.01$) as compared to patients in control group.

RNA Isolation and Real-Time Quantitative RT-PCR

The renal tissue was homogenized, total RNA was extracted using a StrataPrep® Total RNA Microprep Kit (Stratagene, La Jolla, Calif., USA) and reverse transcribed into complementary DNA (cDNA), as described elsewhere [23]. cDNA was amplified by real-time quantitative polymerase chain reaction (PCR) (TaqMan™, ABI Prism 5700 Sequence Detection System, Perkin-Elmer) using fluorogenic probes. To exclude cross-reactivity with genomic DNA, amplification primers were designed to span the exon borders. Samples were tested for genomic DNA contamination and, if tested positive, excluded from the study. TGF-β_1, TNF-α, IL-6, IL-10, MCP-1 and RANTES mRNA were measured in duplicates for each sample. All primers and probes were designed, and assays were validated at the Institute of Medical Immunology, University Medicine Berlin – Charité, Germany. Because preceding experiments demonstrated amplification efficiencies in our system of nearly 1 for all panels, specific gene expression was calculated relative to that of the housekeeping gene HPRT (comparative threshold cycle method ($2^{-\Delta cT}$)).

Statistics

For comparison of data obtained in both groups, the Mann-Whitney test was used. TGF-β, TNF-α, IL-6, IL-10, MCP-1 and RANTES mRNA expression levels were correlated with clinical and morphological data (Spearman-rank correlation) and for binary variables the Mann-Whitney test was used. The receiver-operating characteristic (ROC) curve was used for setting the cutoff points of intrarenal mRNA expression levels of studied genes with the best combination of sensitivity and specificity that indicated the renal graft dysfunction within 42 months after biopsy. Renal outcome was also assessed by Kaplan-Meier survival analysis with log-rank testing. Data are expressed as mean ± SD and $p < 0.05$ was considered to be statistically significant.

Results

Intrarenal Gene Expression

Patients with biopsy-proven CAN exhibited significantly higher expression of all measured genes compared to the control group (fig. 1). Patients with CAN revealed 1.8 times higher TGF-β, 1.5 times higher TNF-α, 4.0 times higher IL-6, 12.0 times higher IL-10, 2.4 times higher MCP-1 and 2.8 times higher RANTES intragraft mRNA expression compared to the control group. The expression levels of followed genes did not differ between groups with different immunosuppressive regimen (data not shown). In the CAN group, there was not any difference in intragraft cytokines and chemokines gene expression levels between the protocol and case biopsies (data not shown).

Table 2. Patient histomorphological data

	Patients	Banff grade				
		0	1	2	3	NC
Glomerulitis (g)	Controls	29	0	0	0	1
	CAN	32	8	1	0	3
Glomerulopathy (cg)	Controls	28	1	0	0	1
	CAN	29	9	2	1	3
Mesangial matrix increase (mm)	Controls	24	5	0	0	1
	CAN	27	10	3	1	3
Tubulitis (t)	Controls	17	10	0	0	3
	CAN	11	33	0	0	0
Tubular atrophy (ct)	Controls	4	23	0	0	3
	CAN	0	24	10	9	1
Interstitial inflammation (i)	Controls	28	0	0	0	2
	CAN	41	2	0	0	1
Interstitial fibrosis (ci)	Controls	27	1	0	0	2
	CAN	0	19	13	10	2
Arteriolar hyaline thickening (ah)	Controls	7	18	3	0	2
	CAN	2	15	13	12	2
Intimal arteritis (v)	Controls	29	0	0	0	1
	CAN	40	0	0	1	3
Vascular fibrous intimal thickening (cv)	Controls	18	8	3	0	1
	CAN	3	15	19	3	4
CAN grade	CAN	0	22	13	9	0

NC = Not classified.

Correlation of Intrarenal Gene Expression with Clinical Parameters

The expression levels of these cytokines and chemokines were compared with clinical data of kidney graft recipients (table 3). We found a significant negative correlation between donor age and IL-10 mRNA expression ($p < 0.05$). The mRNA expression of TNF-α and RANTES correlated with the time post-transplant ($p < 0.05$). Interestingly, the mRNA expression levels of all followed genes correlated with proteinuria. There were significant relations between IL-6 ($p < 0.001$), IL-10 ($p < 0.01$), TNF-α and MCP-1 ($p < 0.05$) and proteinuria, and a trend towards the higher expression of TGF-β_1 and RANTES in patients with higher proteinuria. Moreover, the TGF-β_1 mRNA expression correlated with serum cholesterol levels ($p < 0.05$), MCP-1 mRNA expression with serum creatinine ($p < 0.05$), TNF-α mRNA expression with number of mismatches in HLA-A ($p < 0.05$), and RANTES mRNA expression with number of mismatches in HLA-B ($p < 0.05$). Patients with diabetes mellitus had significantly lower TGF-β_1 mRNA expression than non-diabetic patients ($p < 0.01$). There was no correlation between any cytokine or chemokine mRNA expression level and

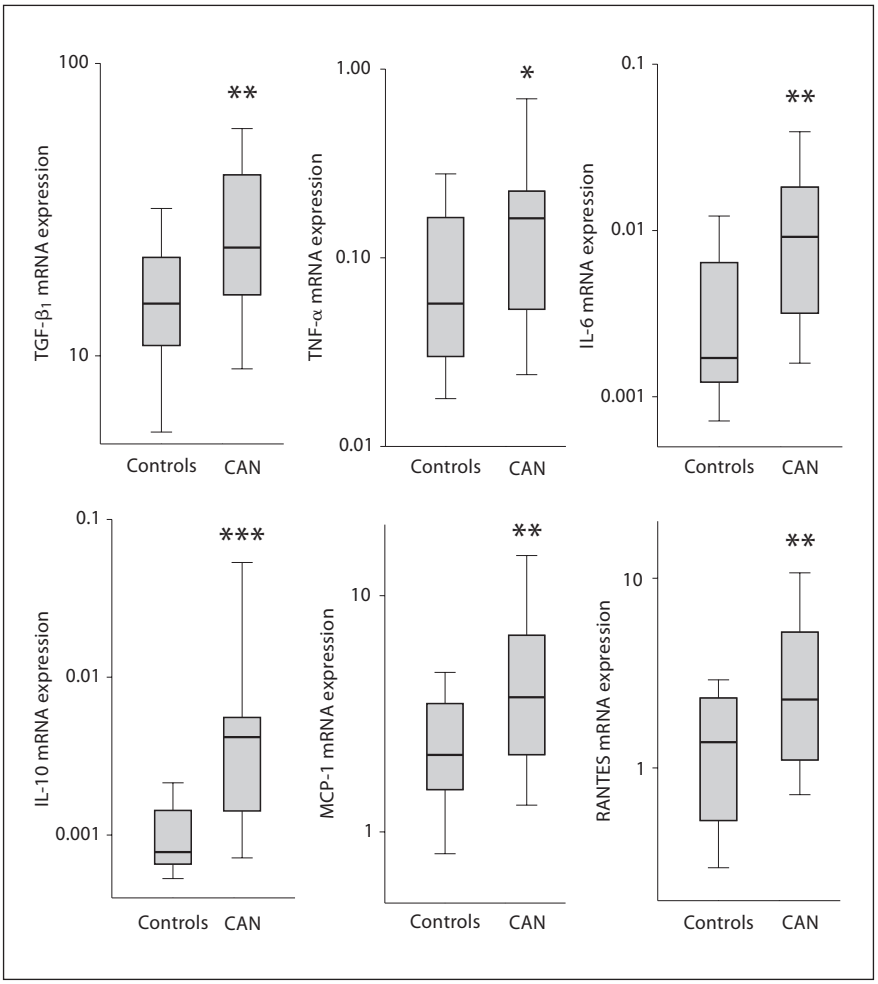

Fig. 1. Quantitative analysis of TGF-β_1, TNF-α, IL-6, IL-10, MCP-1, and RANTES mRNA expression in CAN compared to controls. The results are expressed as the ratio to the housekeeping gene HPRT ($2^{-\Delta cT}$ method). The box plots show 25 and 75th (boundaries of boxes), 50th (median), 10th and 90th (error bars) percentile values. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

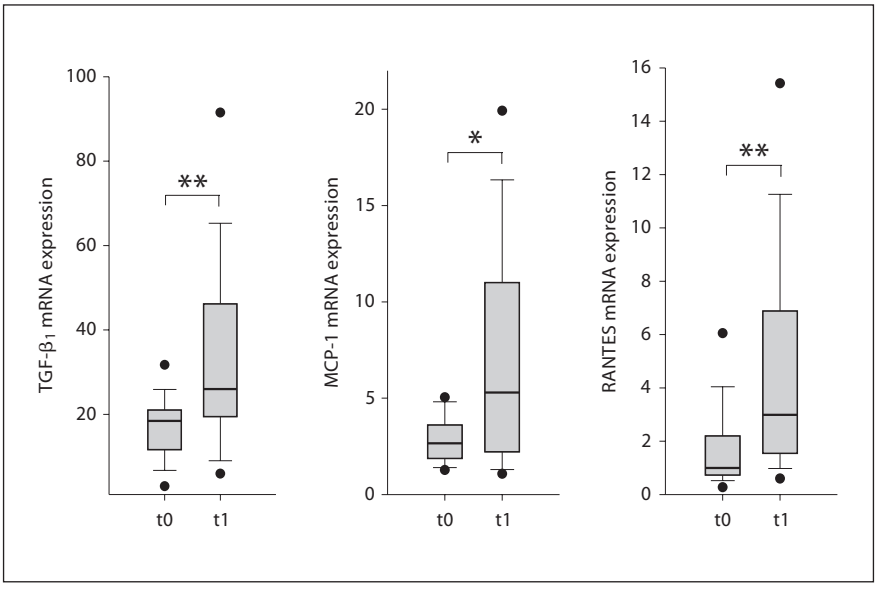

Fig. 2. Comparison of TGF-β_1, MCP-1 and RANTES mRNA expression in CAN between patients with no and mild tubulitis. The results are expressed as the ratio to the housekeeping gene HPRT ($2^{-\Delta cT}$ method). The box plots show 25 and 75th (boundaries of boxes), 50th (median), 10 and 90th (error bars), 5 and 95th (●) percentile values. * $p < 0.05$; ** $p < 0.01$.

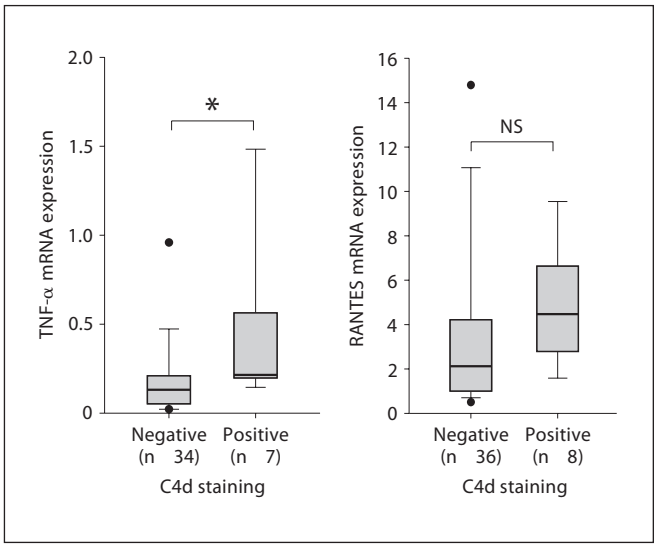

Fig. 3. Comparison of TNF-α and RANTES mRNA expression in CAN between patients with negative and positive C4d staining. The results are expressed as the ratio to the housekeeping gene HPRT ($2^{-\Delta cT}$ method). The box plots show 25 and 75th (boundaries of boxes), 50th (median), 10 and 90th (error bars), 5 and 95th (●) percentile values. * $p < 0.05$; NS = not significant.

Table 3. Correlation between cytokine and chemokine gene expression and clinical data of kidney graft recipients suffering from CAN

Variables[1]	Cytokine/ chemokine	r_s value[2]	p value
Donor age	IL-10	**–0.4525**	**0.0381**
Post-transplant period to biopsy	TNF-α	**0.3670**	**0.0188**
	IL-6	0.3797	0.0528
	RANTES	**0.3032**	**0.0468**
Proteinuria	TGF-β_1	0.2918	0.0556
	TNF-α	**0.3687**	**0.0182**
	IL-6	**0.6050**	**0.0020**
	IL-10	**0.4818**	**0.0273**
	MCP-1	**0.3395**	**0.0278**
	RANTES	0.2863	0.0604
Cholesterol	TGF-β_1	**0.3115**	**0.0411**
Serum creatinine	MCP-1	**0.3070**	**0.0467**
HLA-A mismatches	TNF-α	**0.3126**	**0.0480**
HLA-B mismatches	MCP-1	0.2947	0.0591
	RANTES	**0.3613**	**0.0192**
Diabetes	TGF-β_1		**0.0061**[3]

[1] Only variables where the correlation reached statistical significance for some of followed genes are mentioned.
[2] Spearman rank correlation coefficient. Statistically significant values are represented in boldface type.
[3] Mann-Whitney test.

Table 4. Correlation between cytokine and chemokine gene expression and histomorphological categories used in the Banff working classification of renal allograft biopsies and CAN grade

Variables[1]	Cytokine/ chemokine	r_s value[2]	p value
Glomerulopathy (cg)	IL-6	**0.4781**	**0.0168**
Tubulitis (t)	TGF-β_1	**0.4113**	**0.0070**
	MCP-1	**0.3274**	**0.0338**
	RANTES	**0.4154**	**0.0065**
Tubular atrophy (ct)	MCP-1	**0.3093**	**0.0477**
	RANTES	0.2563	0.0968
Interstitial fibrosis (ci)	MCP-1	0.2613	0.0985
	RANTES	0.3030	0.0524
Arteriolar hyaline thickening (ah)	TGF-β_1	0.2716	0.0820
	IL-6	0.3380	0.0978
Vascular fibrous intimal thickening (cv)	IL-10	–0.3916	0.0967
The sum of 'c' score[3]	TGF-β_1	0.2724	0.0741
	MCP-1	0.2937	0.0570
	RANTES	**0.3126**	**0.0404**
CAN grade	TGF-β_1	0.2681	0.0787
	MCP-1	0.2962	0.0549
	RANTES	0.2688	0.0780

[1] Only variables where the correlation reached statistical significance for some of followed genes are mentioned.
[2] Spearman rank correlation coefficient. Statistically significant values are represented in boldface type.
[3] The sum of categories referring chronic histological changes.

variables such as the age of the patient, gender, the origin of kidney graft, maximal panel-reactive antibodies, plasma triglyceride levels, glomerular filtration rate (GFR), and the incidence of acute rejection and delayed graft function.

Correlation of Intrarenal Gene Expression with Histomorphological Parameters

Cytokine and chemokine expression levels were also correlated with histomorphological categories used in the Banff working classification of renal allograft biopsies and CAN grade (table 4). Interestingly, TGF- β_1 mRNA expression correlated strongly with the degree of tubulitis ($p < 0.01$). Similarly, there were correlations between tubulitis and RANTES ($p < 0.01$) and MCP-1 ($p < 0.05$) mRNA expressions. There were significant differences in expression of these genes between patients without or with mild tubulitis (fig. 2). The occurrence of mild tubulitis in CAN did not differ between protocol and case biopsies (data not shown). MCP-1 mRNA expression cor-

Table 5. Cutoff value for gene mRNA expression determined using the ROC curve analysis

a Controls

Variable	Cutoff value[1, 2]	Sensitivity	Specificity	AUC	95% CI
TGF-β_1	23.0	40.0	93.3	0.647	0.432–0.825
TNF-α	0.035	100.0	60.0	0.780	0.571–0.919
IL-6	0.016	62.5	66.7	0.556	0.301–0.790
IL-10	0.007	71.4	71.4	0.653	0.361–0.878
MCP-1	2.1	70.0	60.0	0.633	0.419–0.815
RANTES	0.53	100.0	46.7	0.720	0.506–0.879

b Patients with CAN

Variable	Cutoff value[1]	Sensitivity	Specificity	AUC	95% CI
TGF-β_1	25.0	54.2	100.0	0.667	0.460–0.834
TNF-α	0.18	56.5	100.0	0.565	0.358–0.757
IL-6	0.045	70.6	100.0	0.735	0.486–0.907
IL-10	0.0013	78.6	50.0	0.536	0.276–0.781
MCP-1	5.2	47.8	100.0	0.565	0.358–0.757
RANTES	1.8	45.8	100.0	0.611	0.406–0.791

[1] Intrarenal gene expression higher than these cutoff values were associated with deteriorated renal function (GFR <0.8 ml/s) at 42 months after the initial biopsy.

[2] The cutoff values are significant for TNF-α and RANTES.

Table 6. Influence of cytokine and chemokine gene overexpression in patients with CAN on graft failure in the long term

Variable	OR	95% CI	p
TGF-β_1 >25 (n = 19)	3.15	0.86–11.05	0.078
MCP-1 >5.2 (n = 14)	5.1	1.28–20.68	0.017

MCP-1 overexpression (ratio MCP-1 vs. HPRT >5.2) in initial biopsies increased the risk (odds ratio) for renal allograft failure within 42 months. χ^2 contingency table analysis.

Table 7. Influence of proteinuria ≥1 g/l on the graft failure

	Odds ratio	95% CI
Graft failure within:		
24 months	6.55	1.57–27.2
36 months	5.37	1.48–19.4
42 months	14.06	3.8–52.0

Patients with proteinuria ≥1 g/l are at a higher risk for graft failure within 24, 36 and 42 months, respectively, than patients with proteinuria <1 g/l. Contingency table analysis.

related with the score of tubular atrophy ($p < 0.05$), and RANTES mRNA expression correlated with the sum of categories referring chronic histological changes ($p < 0.05$). IL-6 mRNA expression correlated with glomerulopathy score ($p < 0.05$). No significant correlation was found between followed cytokine and chemokine mRNA expression and neither interstitial fibrosis of kidney graft nor the grade of CAN.

The Mann-Whitney analysis revealed that TNF-α mRNA expression differed significantly between the biopsies with positive and negative C4d staining ($p < 0.05$). There was a trend towards higher RANTES mRNA expression in patients with positive C4d staining (fig. 3), but there were no differences in expression of other followed genes.

42-Month Follow-Up

Renal allograft recipients were followed for the next 42 months after the initial core biopsy. The ROC curve analysis in controls revealed that TNF-α mRNA expression over 0.035 (TNF- α /HPRT gene expression ratio) was associated with deteriorated renal function (GFR <0.8 ml/s) at 42 months after the initial biopsy (table 5a). Enhanced MCP-1 gene expression in the initial biopsy implied an increased risk for renal graft failure in CAN patients within 42 months: odds ratio 5.1 ($p = 0.017$, table 6), although the ROC curve analysis did not reveal the association of enhanced MCP-1 expression with deteriorated renal function (GFR <0.8 ml/s) at 42 months after the initial biopsy (table 5b). Similarly, there was also a trend towards higher risk for renal graft failure within 42 months after biopsy in grafts with enhanced TGF-β1 expression: odds ratio 3.15 ($p = 0.078$, table 6).

Patients with proteinuria >1 g/l at the time of biopsy were found to be at higher risk for graft dysfunction within 24, 36 and 42 months, respectively, than patients with proteinuria < 1 g/l (table 7).

Kaplan-Meier Analysis of Graft Survival

The Kaplan-Meier analysis revealed that kidney graft recipients with the proteinuria ≥1 g/l at the time of biopsy had significantly shorter graft survival than recipients with the proteinuria <1 g/l ($p < 0.001$, fig. 4). Similarly, patients with CAN and enhanced intrarenal expression of TGF-β1 and MCP-1 at the time of biopsy (ratio to

HPRT ≥25 and 5.2, respectively) had significantly shorter graft survival than patients with the low expression of these genes (fig. 5).

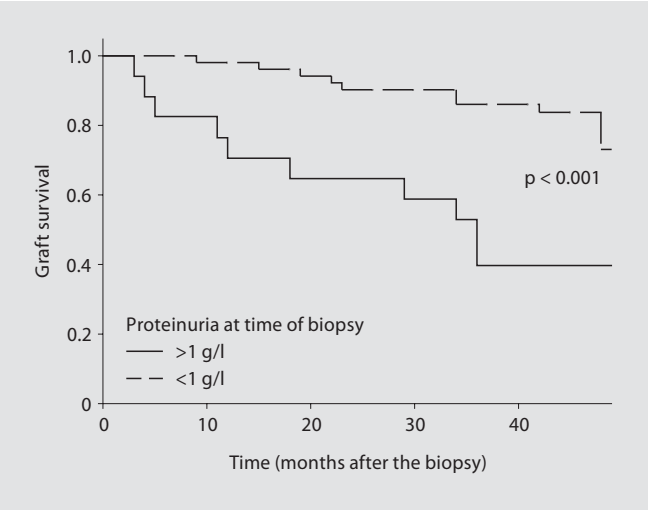

Fig. 4. Kaplan-Meier analysis of influence proteinuria on graft survival.

Discussion

Despite the fact that the short-term results of renal transplantation have significantly improved in recent past years, the long-term survival of kidney graft has remained almost unchanged [1–3]. CAN, according to the Banff 97 classification including IF/TA along with vasculopathy and glomerulopathy, has been shown to be present in the majority of protocol biopsies performed 12 months after transplantation [24]. Similarly to native nephropathies, proteinuria has been suggested to be an important risk factor for kidney graft failure [25–27]. Herein we studied intragraft growth factors, cytokine and chemokine gene expression, and correlated their patterns with the clinical and histomorphological data.

We found all studied cytokines and chemokines to be up-regulated in biopsies revealing CAN. The most up-regulated cytokine in patients suffering from CAN was IL-10. This cytokine was shown to be up-regulated in acute allograft rejection [20, 28]. The up-regulation of intragraft IL-10 expression in CAN might result from endogenous counter-regulation to chronic inflammatory stimuli. It is known that this cytokine, as well as other type 2 cytokines, can promote B-cell proliferation and differentiation into plasma cells, increase antibody production as well as drive a shift from Th1- toward Th2-cell responses. It has been speculated that type 2 cytokines produced by activated macrophages, NK cells, and CD8+ T cells within the graft contribute to the activation of B cells, playing a role in chronic humoral rejection [29].

Recent publications point to a significant role of some cytokines and growth factors, such as TGF-β_1, TNF-α, IL-1β, and IL-6, in the vascular remodeling [30]. TGF-β_1 is a profibrotic factor that may be responsible for the accumulation of extracellular matrix proteins, probably by inducing the synthesis of collagens and inhibiting the synthesis of metalloproteinases, and so contributes to interstitial fibrosis. Many studies demonstrated the higher expression levels of this factor in chronically rejected renal allografts [31–33].

In the present study, the correlation analysis revealed no association of TGF-β_1 mRNA expression level with interstitial fibrosis, glomerulopathy, tubular atrophy, and vascular fibrous intimal thickening. However, higher TGF-β_1 expression levels were associated with mild tubulitis according to the Banff 97 classification. Recently, the TGF-β expression in allograft rejection was shown to be located mainly in the tubular epithelium and, occasionally, on basolateral surface of a tubule and in inflammatory infiltrating cells and its expression increased with tubulitis score. In deteriorating grafts, TGF-β_1 was present within both tubules and interstitial cells [34]. Similar results were found for CC chemokines – higher grades of allograft rejection (with severe tubulitis) were associated with higher expression levels of MCP-1, RANTES, MIP-1β and MIP-1α in tubular epithelium [35]. This corresponds with our results showing a correlation between MCP-1 and RANTES expression and mild tubulitis occurring in CAN according to the Banff 97 classification. Other tests performed on miniature swine revealed the persistent rejection with tubulitis, disrupted tubular basement membrane and tubular atrophy resulting in progressive interstitial fibrosis together with chronic allograft glomerulopathy and arteriopathy in association with the development of chronic rejection [36]. In our study, the occurrence of mild tubulitis in CAN proven by either case or protocol biopsy is in line with the report of Nankivell et al. [24] showing that tubulitis and subclinical rejection were uncovered in 26% of kidney graft protocol biopsies at 12 months after transplantation. All the above findings indicate the important role of tubulitis and TGF-β_1, MCP-1 and RANTES expression in the pathogenesis of CAN.

In almost all chronic renal diseases interstitial fibrosis describes renal function better than any other pathologi-

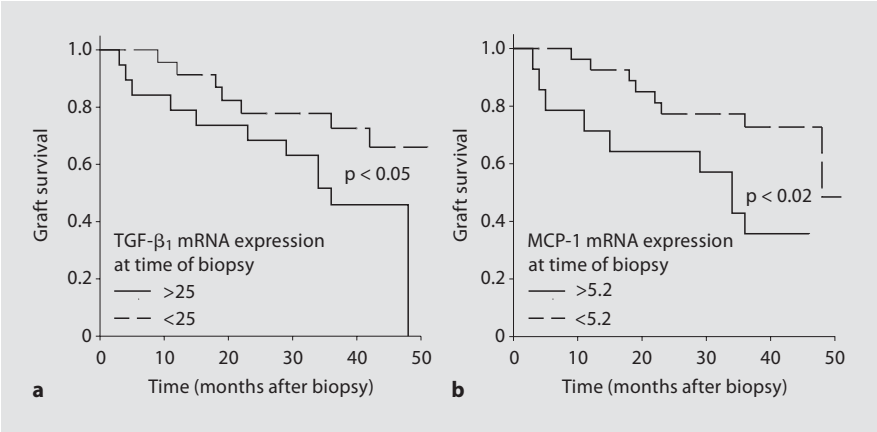

Fig. 5. Kaplan-Meier analysis of variables on renal graft outcome in patients with CAN. **a** Intragraft TGF-β_1 mRNA (log-rank statistic 4.27; p = 0.04). **b** Intragraft MCP-1 mRNA (log-rank statistic 5.62; p = 0.018).

cal finding, and predicts the progression of chronic renal failure. Glomerular proteinuria is a risk factor for the progression of chronic renal failure and interstitial fibrosis [37–41]. In glomerular proteinuria, not only albumin and other 'bulk' plasma proteins, but also growth factors and cytokines (e.g. insulin-like growth factor I, hepatocyte growth factor (HGF), and TGF-β_1) have been shown to be translocated into proximal tubular fluid and activate tubular cells which respond with increased extracellular matrix production. In response to ultrafiltered growth factors, tubular cells also secrete compounds that mediated interactions with the interstitium. CC chemokines (MCP-1, RANTES) contribute to interstitial macrophage accumulation and induce increased TGF-β_1 expression [42–44]. In the present study, we found the correlation between proteinuria and the expression of all followed cytokines and chemokines. Thus, it seems reasonable to hypothesize that mechanisms initialized by proteinuria and growth factors ultrafiltration could cause the up-regulation of inflammatory cytokines and growth factors leading to interstitial fibrosis of transplanted kidney. Because this hypothesis was not supported by any experiment demonstrating a cause and effect, we also analyzed retrospectively the influence of enhanced cytokine and chemokine intrarenal gene expression on the renal graft function in the long term. The up-regulation of MCP-1 and TGF-β_1 was shown to heighten the risk for renal graft failure within 42 months. This finding supplements the results of our previous study that showed high intragraft TGF-β_1 mRNA expression in CAN to represent the risk for worse long-term renal function [45]. Also in control group with normal morphological findings, the increased intrarenal expression of TNF-α was associated with deteriorated renal function. Moreover, Kaplan-Meier graft survival analyses in patients with CAN revealed that enhanced intrarenal expression of TGF-β_1 and MCP-1 at the time of biopsy had a significant influence on the graft survival. Based on the above-mentioned facts, we can recommend the tight monitoring of kidney transplant recipients with the increased intrarenal expression of proinflammatory genes. Patients whose renal graft failed within 42 months after the biopsy had also at the time of biopsy significantly higher proteinuria. The influence of proteinuria $\geq$1 g/l on the renal graft failure was observed as early as at 24 months after biopsy and the Kaplan-Meier graft survival analysis revealed patients with proteinuria $\geq$1 g/l to have inferior graft outcome than those with proteinuria <1 g/l. These findings correspond with others [46, 47] and indicate the proteinuria to be an important modifying factor which must require therapeutic intervention.

A possible limitation of our study could arise from the fact that the patients with normal morphological findings in biopsy had shorter post-transplant follow-up to biopsy compared to patients with CAN. However, it is nearly impossible to obtain a control group with normal morphological findings in kidney allografts later than 12 months after transplantation, since the incidence of CAN is rather high at 1 year after transplantation [24]. As another limitation of the study could be considered that no immunohistochemistry was performed to confirm our data. However, the importance of the correspondence of immunohistochemical and PCR data is not quite unambiguous. Although in some experiments dealing with cytokine, chemokine or cell marker expression the significant correlation was found between mRNA and protein levels [48] or the similar results were obtained using RT-PCR and immunohistochemistry [49, 50], other studies did not find a correlation between mRNA and protein levels [45, 51].

In conclusion, the kidney grafts that suffer from CAN according to the Banff 97 classification differ in the pro-inflammatory cytokine and chemokine gene expression which influence the long-term graft survival. Therefore, these expression patterns rather than the degree of fibrosis may serve as surrogate markers discriminating grafts as the risk for failure.

Acknowledgements

We thank Iva Bruzkova for her excellent technical assistance, Helena Filipova, MD, for ultrasound examination during kidney graft biopsies, Eva Honsova, MD, and Ludek Voska, MD, for excellent histomorphological examination, and Tony Dear for English correction. This study was supported by the Institute for Clinical and Experimental Medicine (MZO 00023001).

References

1 Meier-Kriesche HU, Schold JD, Srinivas TR, Kaplan B: Lack of improvement in renal allograft survival despite a marked decrease in acute rejection rates over the most recent era. Am J Transplant 2004;4:378–383.
2 Carpenter CB: Long-term failure of renal transplants: adding insult to injury. Kidney Int Suppl 1995;50:S40–S44.
3 Paul LC: Chronic renal transplant loss. Kidney Int 1995;47:1491–1499.
4 Racusen LC, Solez K, Colvin RB, Bonsib SM, Castro MC, Cavallo T, Croker BP, Demetris AJ, Drachenberg CB, Fogo AB, Furness P, Gaber LW, Gibson IW, Glotz D, Goldberg JC, Grande J, Halloran PF, Hansen HE, Hartley B, Hayry PJ, Hill CM, Hoffman EO, Hunsicker LG, Lindblad AS, Marcussen N, Mihatsch MJ, Nadasdy T, Nickerson P, Olsen TS, Papadimitriou JC, Randhawa PS, Rayner DC, Roberts I, Rose S, Rush D, Salinas-Madrigal L, Salomon DR, Sund S, Taskinen E, Trpkov K, Yamaguchi Y: The Banff 97 working classification of renal allograft pathology. Kidney Int 1999;55:713–723.
5 Solez K, Axelsen RA, Benediktsson H, Burdick JF, Cohen AH, Colvin RB, Croker BP, Droz D, Dunnill MS, Halloran PF: International standardization of criteria for the histologic diagnosis of renal allograft rejection: the Banff working classification of kidney transplant pathology. Kidney Int 1993; 44:411–422.
6 Halloran PF: Call for revolution: a new approach to describing allograft deterioration. Am J Transplant 2002;2:195–200.
7 Tejani A, Ho PL, Emmett L, Stablein DM: Reduction in acute rejections decreases chronic rejection graft failure in children: a report of the North American Pediatric Renal Transplant Cooperative Study (NAPRTCS). Am J Transplant 2002;2:142–147.
8 Tullius SG, Tilney NL: Both alloantigen-dependent and -independent factors influence chronic allograft rejection. Transplantation 1995;59:313–318.
9 Chapman JR, O'Connell PJ, Nankivell BJ: Chronic renal allograft dysfunction. J Am Soc Nephrol 2005;16:3015–3026.
10 Adams AB, Larsen CP, Pearson TC, Newell KA: The role of TNF receptor and TNF superfamily molecules in organ transplantation. Am J Transplant 2002;2:12–18.
11 Baan CC, Weimar W: Intragraft cytokine gene expression: implications for clinical transplantation. Transpl Int 1998;11:169–180.
12 Border WA, Noble NA: Transforming growth factor-β in tissue fibrosis. N Engl J Med 1994;331:1286–1292.
13 Noronha IL, Eberlein-Gonska M, Hartley B, Stephens S, Cameron JS, Waldherr R: In situ expression of tumor necrosis factor-α, interferon-γ, and interleukin-2 receptors in renal allograft biopsies. Transplantation 1992;54: 1017–1024.
14 Paul LC, Saito K, Davidoff A, Benediktsson H: Growth factor transcripts in rat renal transplants. Am J Kidney Dis 1996;28:441–450.
15 Hancock WH, Whitley WD, Tullius SG, Heemann UW, Wasowska B, Baldwin WM, 3rd, Tilney NL: Cytokines, adhesion molecules, and the pathogenesis of chronic rejection of rat renal allografts. Transplantation 1993;56:643–650.
16 Nadeau KC, Azuma H, Tilney NL: Sequential cytokine dynamics in chronic rejection of rat renal allografts: roles for cytokines RANTES and MCP-1. Proc Natl Acad Sci USA 1995;92:8729–8733.
17 Azuma H, Heemann UW, Tullius SG, Tilney NL: Cytokines and adhesion molecules in chronic rejection. Clin Transplant 1994;8: 168–180.
18 Song E, Zou H, Yao Y, Proudfoot A, Antus B, Liu S, Jens L, Heemann U: Early application of Met-RANTES ameliorates chronic allograft nephropathy. Kidney Int 2002;61: 676–685.
19 Melk A, Henne T, Kollmar T, Strehlau J, Latta K, Offner G, Jhangri GS, Ehrich JH, Von Schnakenburg C: Cytokine single nucleotide polymorphisms and intrarenal gene expression in chronic allograft nephropathy in children. Kidney Int 2003;64:314–320.
20 Strehlau J, Pavlakis M, Lipman M, Shapiro M, Vasconcellos L, Harmon W, Strom TB: Quantitative detection of immune activation transcripts as a diagnostic tool in kidney transplantation. Proc Natl Acad Sci USA 1997;94:695–700.
21 Zegarska J, Paczek L, Pawlowska M, Podrzucki W, Rowinski W, Malanowski P, Wszola M, Mroz A: Quantitative gene expression of TGF-β_1, TNF-α, IL-1β, and IL-6 in the renal artery wall of chronically rejected human renal allografts. Transplant Proc 2002; 34:3176–3179.
22 Cockcroft DW, Gault MH: Prediction of creatinine clearance from serum creatinine. Nephron 1976;16:31–41.
23 Platzer C, Ode-Hakim S, Reinke P, Docke WD, Ewert R, Volk HD: Quantitative PCR analysis of cytokine transcription patterns in peripheral mononuclear cells after anti-CD3 rejection therapy using two novel multispecific competitor fragments. Transplantation 1994;58:264–268.
24 Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Allen RD, Chapman JR: The natural history of chronic allograft nephropathy. N Engl J Med 2003;349:2326–2333.
25 Arias M, Fernandez-Fresnedo G, Rodrigo E, Ruiz JC, Gonzalez-Cotorruelo J, Gomez-Alamillo C: Non-immunologic intervention in chronic allograft nephropathy. Kidney Int Suppl 2005:S118–S123.
26 Joosten SA, Sijpkens YW, van Kooten C, Paul LC: Chronic renal allograft rejection: pathophysiologic considerations. Kidney Int 2005; 68:1–13.
27 Massy ZA, Guijarro C, Wiederkehr MR, Ma JZ, Kasiske BL: Chronic renal allograft rejection: immunologic and non-immunologic risk factors. Kidney Int 1996;49:518–524.
28 Suthanthiran M: Molecular analyses of human renal allografts: differential intragraft gene expression during rejection. Kidney Int Suppl 1997;58:S15–S21.
29 Orosz CG: Considerations regarding the contributions of B cells to chronic allograft rejection in experimental animal models. J Heart Lung Transplant 2000;19:634–637.
30 Paul LC: Chronic allograft nephropathy: an update. Kidney Int 1999;56:783–793.

31 Sharma VK, Bologa RM, Xu GP, Li B, Mouradian J, Wang J, Serur D, Rao V, Suthanthiran M: Intragraft TGF- β 1 mRNA: a correlate of interstitial fibrosis and chronic allograft nephropathy. Kidney Int 1996;49:1297–1303.

32 Sharma VK, Ding R, Li B, Bologa RM, Lagman M, Eduafo A, Edouard P, Mouradian J, Strom TB, Suthanthiran M: Molecular correlates of human renal allograft rejection. Transplant Proc 1998;30: 2364–2366.

33 Shihab FS, Yamamoto T, Nast CC, Cohen AH, Noble NA, Gold LI, Border WA: Transforming growth factor- β and matrix protein expression in acute and chronic rejection of human renal allografts. J Am Soc Nephrol 1995;6: 286–294.

34 Robertson H, Wong WK, Talbot D, Burt AD, Kirby JA: Tubulitis after renal transplantation: demonstration of an association between CD103+ T cells, transforming growth factor- β 1 expression and rejection grade. Transplantation 2001;71:306–313.

35 Robertson H, Morley AR, Talbot D, Callanan K, Kirby JA: Renal allograft rejection: β-chemokine involvement in the development of tubulitis. Transplantation 2000;69: 684–687.

36 Shimizu A, Yamada K, Sachs DH, Colvin RB: Persistent rejection of peritubular capillaries and tubules is associated with progressive interstitial fibrosis. Kidney Int 2002; 61:1867–1879.

37 Peterson JC, Adler S, Burkart JM, Greene T, Hebert LA, Hunsicker LG, King AJ, Klahr S, Massry SG, Seifter JL: Blood pressure control, proteinuria, and the progression of renal disease. The Modification of Diet in Renal Disease Study. Ann Intern Med 1995; 123:754–762.

38 Remuzzi G, Bertani T: Pathophysiology of progressive nephropathies. N Engl J Med 1998;339: 1448–1456.

39 Ruggenenti P, Perna A, Mosconi L, Matalone M, Pisoni R, Gaspari F, Remuzzi G: Proteinuria predicts end-stage renal failure in non-diabetic chronic nephropathies. The 'Gruppo Italiano di Studi Epidemiologici in Nefrologia' (GISEN). Kidney Int Suppl 1997; 63:S54–S57.

40 Sheerin NS, Sacks SH: Chronic interstitial damage in proteinuria. Does complement mediate tubulointerstitial injury? Kidney Blood Press Res 1999;22:47–52.

41 Wang S, LaPage J, Hirschberg R: Proteinuria and progression of chronic renal disease. Kidney Blood Press Res 2000; 23: 167–169.

42 Iwano M, Neilson EG: Mechanisms of tubulointerstitial fibrosis. Curr Opin Nephrol Hypertens 2004; 13: 279–284.

43 Wang SN, LaPage J, Hirschberg R: Role of glomerular ultrafiltration of growth factors in progressive interstitial fibrosis in diabetic nephropathy. Kidney Int 2000; 57:1002–1014.

44 Abbate M, Remuzzi G: Proteinuria as a mediator of tubulointerstitial injury. Kidney Blood Press Res 1999;22: 37–46.

45 Pribylova-Hribova P, Kotsch K, Loderer-ova A, Viklicky O, Vitko S, Volk HD, Lacha J: TGF- β 1 mRNA upregulation influences chronic renal allograft dysfunction. Kidney Int 2006; 69:1872–1879.

46 Hohage H, Kleyer U, Bruckner D, August C, Zidek W, Spieker C: Influence of proteinuria on long-term transplant survival in kidney transplant recipients. Nephron 1997; 75:160–165.

47 McLaren AJ, Fuggle SV, Welsh KI, Gray DW, Morris PJ: Chronic allograft failure in human renal transplantation: a multivariate risk factor analysis. Ann Surg 2000; 232:98–103.

48 Diegmann J, Junker K, Gerstmayer B, Bosio A, Hindermann W, Rosenhahn J, von Eggeling F: Identification of CD70 as a diagnostic biomarker for clear cell renal cell carcinoma by gene expression profiling, real-time RT-PCR and immunohistochemistry. Eur J Cancer 2005; 41:1794–1801.

49 Gu B, Lu Y, Xin YP, Zhang XH, Wang J, Li YP: The effects of different immunosuppressants on chronic allograft nephropathy by affecting the transforming growth factor-β and Smads signal pathways. Transplant Proc 2006;38: 2154–2157.

50 Mu W, Ouyang X, Agarwal A, Zhang L, Long DA, Cruz PE, Roncal CA, Glushakova OY, Chiodo VA, Atkinson MA, Hauswirth WW, Flotte TR, Rodriguez-Iturbe B, Johnson RJ: IL-10 suppresses chemokines, inflammation, and fibrosis in a model of chronic renal disease. J Am Soc Nephrol 2005; 16:3651–3660.

51 Kim TW, Ji JW, Chang HG, Kim MO, Ryoo ZY, Park IK, Kim SJ: 3′-Half of the thrombopoietin cDNA confers higher expression of erythropoietin at the RNA level but not at the protein level. Mol Cells 2005;19:198–204.

Genetic Variability of Major Inflammatory Mediators Has No Impact on the Outcome of Kidney Transplantation

Irena Brabcova,[1] Jan Petrasek,[2] Petra Hribova,[1] Katerina Hyklova,[1] Katerina Bartosova,[3] Jiri Lacha,[1,3] and Ondřej Viklický[1,3,4]

Background. Functionally relevant polymorphisms in genes of the Th1 and Th2-inflammatory pathway influence the susceptibility to acute rejection (AR), chronic allograft nephropathy (CAN), and subclinical rejection (SR) as well as graft survival after renal transplantation. Because these findings have not been validated, we sought confirmatory evidence of these associations in a larger group of renal transplant recipients.

Methods. A total of 436 kidney transplant recipients were genotyped for 9 single nucleotide polymorphisms (*TNF-α*-308G/A, *MCP-1*-2518A/G, *RANTES*-403G/A, −109T/C and −28C/G, *CCR2+190G/A*, *IFN-γ*+874A/T, *TGF-β+869T/C* and *+915G/C*) and for the 32-bp indel polymorphism in *CCR5*. The effects of these polymorphisms on the incidence of AR, SR, CAN and graft survival were analyzed in single locus and haplotype models.

Results. Single locus analysis revealed that there was no significant difference in the distribution of the genotype frequencies between patients with and without AR, and between patients with CAN or SR, and individuals without CAN. Furthermore, no influence of any of the polymorphisms on the long-term graft survival was observed. Haplotype [*TGF-β* +869G; *TGF-β* +915C] seemed to be associated with the presence of SR (odds ratio: 3.45, 95% confidence interval: 1.19 – 9.99, $P=0.023$), but the association was nonsignificant due to the insufficient power.

Conclusion. In contrast to previous allelic association studies, neither of the polymorphisms has been associated with the outcome of kidney transplantation in the single locus analysis nor in the haplotype model. Our findings reinforce the need for more rigorous research compliant with the currently accepted standards for polymorphism-disease association studies.

Keywords: Cytokines, Gene polymorphisms, Chronic allograft nephropathy, Acute rejection, Graft function.

(*Transplantation* 2007;84: 1037–1044)

Acute and chronic renal graft rejection influences the results of kidney transplantation and thus remains the major obstacle in the success of renal transplantation. In addition to human leukocyte antigen loci, several genes encoding cytokines and their receptors have been recently suggested to play an important role in the process of rejection (*1–3*).

As the pathogenesis of rejection is complex, there are many candidate genes that could potentially influence individual susceptibility. Tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β), interferon gamma (IFN-γ), regulated upon activation normal T-cell expressed and secreted (RANTES), monocyte chemotactic protein-1 (MCP-1), CCR2, and CCR5 receptors have been all shown to be involved in pathogenesis of renal allograft rejection in experimental setting.

Animal models of acute and chronic kidney allograft rejection showed an increased intragraft expression of *TGF-β* (*4*), *TNF-α*, *IFN-γ*, *RANTES,* and *MCP-1* (*5*) compared to control animals without rejection. In a mouse renal interstitial fibrosis model, *Ccr2* and *Ccr5* knockout animals exhibited reduced fibrosis comparable to that of their wild-type littermates (*6*, *7*). Furthermore, overexpression of TGF-β in rats leads to mesangial expansion, accumulation of glomerular matrix proteins, and interstitial fibrosis leading to progressive glomerulosclerosis (*8*).

Although it is well accepted that cytokine-mediated inflammation promotes the development of cellular injury, interstitial fibrosis, and tubular atrophy with subsequent allograft dysfunction, there is less evidence that genetic variability in the cytokines determines the susceptibility for kidney graft rejection.

This work was supported by the Institute for Clinical and Experimental Medicine (MZO 00023001).

[1] Transplant Laboratory, Institute for Clinical and Experimental Medicine, Prague, Czech Republic.
[2] Laboratory of Experimental Hepatology, Institute for Clinical and Experimental Medicine, Prague, Czech Republic.
[3] Department of Nephrology, Institute for Clinical and Experimental Medicine, Prague, Czech Republic.
[4] Address correspondence to: Ondřej Viklický, M.D., Ph.D., Department of Nephrology, Transplant Laboratory, Institute for Clinical and Experimental Medicine, Vídeňská 1958, 140 00 Prague 4, Czech Republic.
E-mail: ondrej.viklicky@ikem.cz

Received 30 May 2007. Revision requested 27 June 2007. Accepted 11 July 2007.

ISSN 0041-1337/07/8408-1037
DOI: 10.1097/01.tp.0000285295.39275.3b

Recently, numerous polymorphisms in the promoter regions of *TNF-α*, *MCP-1*, *RANTES* and in the translated regions of *CCR2*, *CCR5*, *IFN-γ*, and *TGF-β* genes have been identified. In vitro studies showed that some of them influence either the expression of the respective gene or the function of the respective protein. Accordingly, the *TNF-α* −308G/A and the *MCP-1* promoter polymorphisms −2518A/G enhance the production of TNF-α (*9*) and MCP-1, respectively, by monocytes (*10*). Haplotype [− 403A; − 109C; − 28G] in the promoter of *RANTES* increase transcription of the gene (*11*). The polymorphism *CCR2* +190G/A, corresponding to the amino acid exchange Val64Ile in the second exon of the gene, increases the transcription of *CCR2* (*12*), whereas deletion of 32-base pairs in the coding region of *CCR5* results in a frameshift and premature termination of translation at codon 185 (*13*). Polymorphism *IFN-γ* +874A/T is located within a binding site for the transcription factor nuclear factor-κB and causes a significant increase of *IFN-γ* transcription (*14*). Finally, two polymorphisms +869T/C and +915G/C in the coding region of *TGF-β*, corresponding to the amino acid exchange Leu10Pro and Arg25Pro, impeded the production of *TGF-β* by lymphocytes (*15*).

Consequently, the role of these polymorphisms in the susceptibility to the allograft nephropathy in humans has been studied and several groups reported an association with acute and chronic kidney graft rejection (*16–22*). Indeed, these findings could be utilized in the identification of kidney allograft recipients predisposed to allograft rejection and thus potentially benefiting from tailored immunosuppression. However, in view of the small numbers of individuals involved in the studies resulting in inadequate statistical power, and the fact that none of the reported associations has been replicated in an independent sample of patients, we sought to investigate the frequency of these polymorphisms in a large cohort of well-characterized patients who underwent renal transplantation.

PATIENTS AND METHODS

Patient Population

We consecutively included 436 white kidney transplant recipients who had undergone kidney transplantation at the Institute for Clinical and Experimental Medicine, Prague, the Czech Republic, from 1999 to 2004. In the posttransplant course, all patients received either cyclosporine or tacrolimus along with mycophenolate mofetil and steroids, recipients with panel reactive antibodies >50% received prophylaxis by muromonab-OKT3 or anti-thymocyte globulin. All acute rejection episodes (AR) were determined according to Banff 97 criteria (*23*) and were biopsy proven; borderline changes were included.

Clinical and laboratory data were collected on the date that the protocol 12-month biopsy was performed. A complete physical examination was undertaken in all patients. Laboratory evaluation included creatinine, total cholesterol, and total triglycerides. The glomerular filtration rate was estimated by the Cockcroft-Gault formula (*24*).

Renal Histology

The protocol 12-month biopsy was performed in 273 patients with functioning kidney graft out of 436 patients who gave an inform consent with the protocol biopsy. Biopsies was performed using Tru-cut needle (Uni-Cut Nadeln, Angiomed, Germany) guided by ultrasound (Toshiba, Power Vision 6000, Japan). Samples were routinely stained with hematoxylin and eosin, periodic acid-Schiff, aldehyde-fuchsin orange G, Sirius red with elastic stain, and periodic acid silver-methenamine. Biopsy tissues were scored on the basis of the Banff 97 working classification (*23*). Subclinical rejection (SR) was defined as histological findings of AR including borderline changes at 12-month protocol biopsy and stable kidney graft function.

The study was approved by the institutional review board and written informed consent was obtained from all subjects.

Genotyping of Polymorphisms

The genomic DNA was isolated from whole blood samples using a commercial kit (Whole blood DNA purification kit; Fermentas, Canada). Single nucleotide polymorphisms were determined by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism analysis: *RANTES, MCP-1*, *CCR2* (*19*, *25–27*) or by sequence specific priming (PCR-SSP): *TNF-α*, *IFN-γ* and *TGF-β* (*28–30*). The insertion-deletion 32bp polymorphism in *CCR5* was determined by a simple PCR method (*20*).

To minimize genotyping errors, blank controls wells were left on the PCR plates and assays were wholly retyped in the call rate was under 80%. Three operators (I.B., P.H., K.H.) independently performed genotype assignment. Genotyping was duplicated in case of discrepancy between operators. After testing for Hardy-Weinberg equilibrium, allele frequencies were checked for consistency with data from Utah residents with Northern and Western European ancestry (CEU) population of European ancestry from the HapMap database (*31*).

Statistical Analysis

We calculated the sample size required to detect the effect of an polymorphism on the risk of outcome using the DSTPLAN software (http://linkage.rockefeller.edu/soft). When the odds ratio (OR) of a polymorphism was assumed to be 2, the required sample size was 110 cases and 110 controls for the polymorphism with the variant allele frequency of 0.5 (*TGF-β* +915G/C). When the OR was assumed to be 4, the same sample size was sufficient to detect a true effect of a polymorphism with the allele frequency of 0.04 (*RANTES* −28C/G). In a model with 190 patients and 190 controls, the minimal detectable OR values for the same allele frequencies were 1.8 and 3.1, respectively. The calculations have been performed at a 5% level of significance for 80% statistical power.

Hardy-Weinberg equilibrium of alleles at individual loci was evaluated using the chi-square test. Linkage disequilibrium coefficients $D' = D/D_{min\ or\ max}$ and r^2 were calculated using the standard formulas (*32*). The coefficients *D'* and r^2, ranging from 0 to 1, denote the strength of the LD. The value of $D'=1$ indicates a complete LD if one haplotype is not observed and $r^2=1$ indicates a perfect LD in case that two haplotypes are not observed.

To evaluate the effect of continuous variables on the respective clinical outcomes, one-way analysis of variance was used. Single-locus association analyses with calculation of OR and 95% confidence intervals (CI) were performed by univariate logistic regression analysis using SPSS version 14.0 (SPSS Inc., Chicago, IL). Subsequently, multivariate regres-

TABLE 1. Clinical and laboratory parameters in patients at 12-month protocol biopsy

	Chronic allograft nephropathy	Normal biopsy	Subclinical rejection	*P* value
N	122	113	38	
Serum creatinine (μmol/L)	173.0±77.0	130.0±40.8	190.4±166.0	<0.01[a]
Glomerular filtration rate (mL/s)	0.94±0.40	1.11±0.38	0.99±0.36	<0.01[b]
Proteinuria (g/day)	0.60±1.09	0.32±0.47	0.58±0.79	<0.001[b]
Cholesterol (mmol/L)	5.58±1.18	5.47±1.04	5.46±1.14	0.7
Triglycerides (mmol/L)	2.56±1.23	2.38±1.24	2.20±0.75	0.14
Body mass index (kg/m^2)	26.4±4.8	26.4±4.9	26.1±5.4	0.92
Panel reactive antibody (%)	20.93±28.89	20.00±29.37	19.34±24.55	0.53
HLA mismatches	2.95±1.30	2.98±1.24	3.00±1.40	0.97
HLA DR mismatches	0.70±0.67	0.60±0.62	0.82±0.69	0.69
Systolic blood pressure (mm Hg)	145.0±18.5	143.0±19.5	142.6±15.8	0.62
Diastolic blood pressure (mm Hg)	86.8±10.3	85.3±10.6	86.1±10.7	0.55
Acute rejection incidence (%)	35.2	21.1		<0.05[b]
Donor age (years)	46.5±14.9	42.1±16.3	42.4±15.9	0.08
Donor sex (% male)	59.0	54.9	50.0	0.59
Recipient age (years)	46.1±12.4	48.5±12.6	44.6±12.8	0.15
Recipient gender (% male)	65.6	62.3	78.9	0.17

Continuous variables are means±SD.
[a] Significance between all groups.
[b] Significance between chronic allograft nephropathy group compared to normal group.

sion adjusted for total number of mismatches, mismatches in DR locus, and immunosuppression regimen (either cyclosporine or tacrolimus-based) was performed. For haplotype analysis, univariate logistic regression was used. To test the effect of the polymorphisms on the graft survival, the Kaplan-Meier analysis was used. The level of significance was set at $P<0.05$. All *P* values were two-sided.

RESULTS

Subject Characteristics

The age median of transplanted patients was 49.2 (range 19.0–76.0) years; of them, 287 (65.8%) were male. The mean number of mismatches between donor and recipient in human leukocyte antigen (*HLA*)-*A*, -*B,* and -*DR* loci was 3.1±1.2 and mean percentage of panel reactive antibodies was 19.7±27.3. The donor age median was 46.0 (range 2.0–76.0) years.

Upon the 12-month protocol kidney graft biopsy, chronic allograft nephropathy (CAN) was found in 122 (44.5%) patients (CAN grade I in 82 patients, grade II in 27 patients and grade III in 13 patients). Subclinical rejection was present in 38 patients (Table 1). Patients with CAN had higher creatinine level and proteinuria with correspondingly lower glomerular filtration rate values. Moreover, 35% of patients with CAN presented with AR during the first post-transplant year, compared to 21% of patients without CAN. One-hundred and ninety patients with AR had significantly increased creatinine level and proteinuria, compared to those without AR (Table 2). Apart from that, no clinically meaningful differences between the groups were observed.

Genetic Polymorphisms

All alleles at individual loci were in Hardy-Weinberg equilibrium with nonsignificant χ^2 values in all groups. The genotype frequencies in the control groups for all polymorphisms were in concordance with the reference HapMap database (*31*). There was a significant linkage disequilibrium (LD) between the *TGF-β* +869T/C and *TGF-β* +915G/C loci, with 69% of the inferred haplotypes [*TGF-β* +869; *TGF-β* +915]

TABLE 2. Clinical and laboratory parameters in patients with and without acute rejection history at 12 month after transplantation

	Acute rejection	No acute rejection	*P* value
N	190	246	
Serum creatinine (μmol/L)	173.46±98.66	146.53±64.92	<0.01
Glomerular filtration rate (mL/s)	0.96±0.37	1.02±0.39	0.10
Proteinuria (g/day)	0.6±1.02	0.47±1.14	<0.01
Cholesterol (mmol/L)	5.55±1.31	5.59±1.07	0.74
Triglycerides (mmol/L)	2.54±1.23	2.31±1.14	<0.05
Body mass index (kg/m^2)	26.75±5.20	25.99±4.46	0.12
Panel reactive antibody (%)	19.86±26.52	19.59±27.86	0.81
HLA mismatches	3.25±1.20	2.9±1.23	<0.01
HLA DR mismatches	0.79±0.67	0.57±0.63	<0.01
Systolic blood pressure (mm Hg)	139.66±17.22	141.73±18.98	0.25
Diastolic blood pressure (mm Hg)	84.40±10.99	85.04±9.98	0.54
Donor age (years)	44.33±16.99	43.25±15.47	0.49
Donor sex (% male)	51.6	61.6	<0.05
Recipient age (years)	45.82±12.00	47.70±12.72	0.12
Recipient gender (% male)	61.8	69.1	0.11

Continuous variables are means±SD.

consisting of either T-G or C-C. The linkage between these two loci calculated in the CAN-negative control group was almost complete (D'=0.99), but not perfect (r^2=0.11).

Single Locus Analysis

Neither univariate analysis nor multivariate analysis adjusted for number of mismatches, DR-locus haplotype and immunosuppression regimen showed significant difference in the distribution of the genotype frequencies between patients with and without AR (Table 3), and between patients with CAN or SR and individuals with normal 12-month protocol biopsy (Table 4). Furthermore, no influence of any polymorphism on the graft survival was observed (Fig. 1).

TABLE 3. Genotype frequencies in the groups of patients with and without acute rejection history during the first year after transplantation.

	No acute rejection (%)	Acute rejection (%)	Association with acute rejection, odds ratio (95% CI)	*P* value
N	246	190		
MCP-1 −2518				
A/A	139 (57)	105 (55)	1	
A/G	90 (37)	73 (38)	1.09 (0.80–1.50)	0.58
G/G	17 (7)	12 (6)	0.90 (0.47–2.34)	0.90
CCR2 V64I				
V/V	186 (76)	148 (78)	1	
V/I	59 (24)	42 (22)	0.84 (0.55–1.30)	0.44
I/I	1 (0)	0 (0)	—	—
CCR5 del 32 bp				
ins/ins	189 (77)	155 (82)	1	
ins/del	55 (22)	32 (17)	0.80 (0.52–1.26)	0.33
del/del	2 (1)	3 (2)	2.08 (0.32–13.28)	0.44
TNF-α −308				
G/G	181 (74)	138 (73)	1	
G/A	62 (25)	50 (26)	1.03 (0.69–1.53)	0.88
A/A	3 (1)	2 (1)	0.74 (0.11–4.83)	0.75
IFN-γ +874				
T/T	50 (20)	41 (22)	1	
T/A	119 (48)	98 (52)	0.91 (0.69–1.20)	0.48
A/A	77 (31)	51 (27)	0.80 (0.46–1.40)	0.43
RANTES −403				
G/G	163 (66)	118 (62)	1	
G/A	72 (29)	67 (35)	1.10 (0.78–1.56)	0.59
A/A	11 (4)	5 (3)	0.72 (0.24–2.18)	0.56
RANTES −109				
T/T	221 (90)	179 (94)	1	
T/C	25 (10)	11 (6)	0.52 (0.25–1.09)	0.08
C/C	0 (0)	0 (0)	—	—
RANTES −28				
C/C	229 (93)	182 (96)	1	
C/G	16 (7)	8 (4)	0.52 (0.22–1.23)	0.14
G/G	1 (0)	0 (0)	—	—
TGF-β +869, cod 10				
C/C	84 (34)	67 (35)	1	
T/C	128 (52)	91 (48)	1.01 (0.76–1.33)	0.97
T/T	34 (14)	32 (17)	1.09 (0.60–1.07)	0.79
TGF-β +915, cod 25				
G/G	200 (81)	162 (85)	1	
G/C	44 (18)	26 (14)	0.77 (0.47–1.27)	0.31
C/C	2 (1)	2 (1)	0.96 (0.12–7.41)	0.96

Data were adjusted for the total number of mismatches, mismatches in DR locus and immunosuppression regimen in multivariate analysis.

TABLE 4. Genotype frequencies in the groups of patients with and without chronic allograft nephropathy and subclinical rejection findings in 12-month protocol biopsy

Polymorphism	Normal biopsy (%)	Chronic allograft nephropathy (%)	Subclinical rejection (%)	Association with chronic allograft nephropathy		Association with subclinical rejection	
				OR (95% CI)	*P* value	OR (95% CI)	*P* value
N	113	122	38				
MCP-1 −2518							
A/A	68 (60)	65 (53)	18 (47)	1		1	
A/G	36 (32)	49 (40)	18 (47)	1.22 (0.80–1.87)	0.36	1.46 (0.80–2.66)	0.22
G/G	9 (8)	8 (7)	2 (5)	1.00 (0.36–2.80)	0.99	1.07 (0.20–5.61)	0.94
CCR2 V64I							
V/V	85 (75)	95 (78)	25 (66)	1		1	
V/I	28 (25)	26 (21)	13 (34)	0.95 (0.54–1.69)	0.87	1.56 (0.75–3.25)	0.23
I/I	0	1 (1)	0 (0)	—	—	—	—
CCR5 del 32 bp							
ins/ins	92 (81)	98 (80)	29 (76)	1		1	
ins/del	21 (19)	22 (18)	8 (21)	1.13 (0.61–2.09)	0.70	1.36 (0.60–3.08)	0.47
del/del	0	2 (2)	1 (3)	—	—	—	—
TNF-a −308							
G/G	85 (75)	88 (72)	24 (63)	1		1	
G/A	27 (24)	34 (28)	14 (37)	1.14 (0.66–1.97)	0.64	1.65 (0.80–3.37)	0.17
A/A	1 (1)	0 (0)	0 (0)	—	—	—	—
IFN-γ +874							
T/T	22 (19)	29 (24)	5 (13)	1		1	
T/A	54 (48)	61 (50)	19 (50)	0.84 (0.58–1.22)	0.35	1.27 (0.73–2.2)	0.39
A/A	37 (33)	32 (26)	14 (37)	0.74 (0.35–1.56)	0.43	1.98 (0.59–6.60)	0.27
RANTES −403							
G/G	70 (62)	82 (67)	24 (63)	1		1	
G/A	39 (35)	36 (30)	13 (34)	0.92 (0.57–1.46)	0.71	1.04 (0.53–2.03)	0.91
A/A	4 (4)	4 (3)	1 (3)	0.96 (0.23–4.07)	0.96	0.82 (0.09–7.85)	0.86
RANTES −109							
T/T	102 (90)	111 (91)	36 (95)	1		1	
T/C	11 (10)	11 (9)	2 (5)	0.86 (0.36–2.04)	0.73	0.43 (0.09–2.23)	0.29
C/C	0 (0)	0 (0)	0 (0)	—	—	—	—
RANTES −28							
C/C	104 (92)	116 (95)	36 (95)	1		1	
C/G	8 (7)	6 (5)	2 (5)	0.86 (0.71–1.04)	0.18	0.58 (0.12–2.77)	0.58
G/G	1 (1)	0 (0)	0 (0)	—	—	—	—
TGF-β +869, cod 10							
C/C	41 (36)	43 (35)	7 (18)	1		1	
T/C	57 (50)	60 (49)	30 (79)	0.86 (0.71–1.03)	0.99	1.05 (0.61–1.81)	0.86
T/T	15 (13)	19 (16)	1 (3)	1.15 (0.50–2.63)	0.74	0.33 (0.04–2.97)	0.32
TGF-β +915, cod 25							
G/G	100 (88)	103 (84)	28 (74)	1		1	
G/C	11 (10)	18 (15)	10 (26)	1.32 (0.65–2.69)	0.44	2.23 (0.92–5.36)	0.08
C/C	2 (2)	1 (1)	0 (0)	0.52 (0.04–6.09)	0.60	—	—

Data were adjusted for the total number of mismatches, mismatches in DR locus, and immunosuppression regimen in multivariate analysis.

Haplotype Analysis

Haplotype (TGF-β +869G; TGF-β +915C) seemed to be associated with the presence of SR (OR: 3.45, 95% confidence interval: 1.19–9.99, $P=0.023$), but the association was nonsignificant due to the insufficient power (data not shown).

DISCUSSION

In the white population, promoter polymorphisms *TNF-α* −308G/A, *MCP-1* −2518A/G, *RANTES* −403G/A, −109T/C and −28C/G, and exonal polymorphisms *CCR2*+*190G/A*, *CCR5* del32bp, *IFN-γ* +874A/T, *TGF-β* +*869T/C*

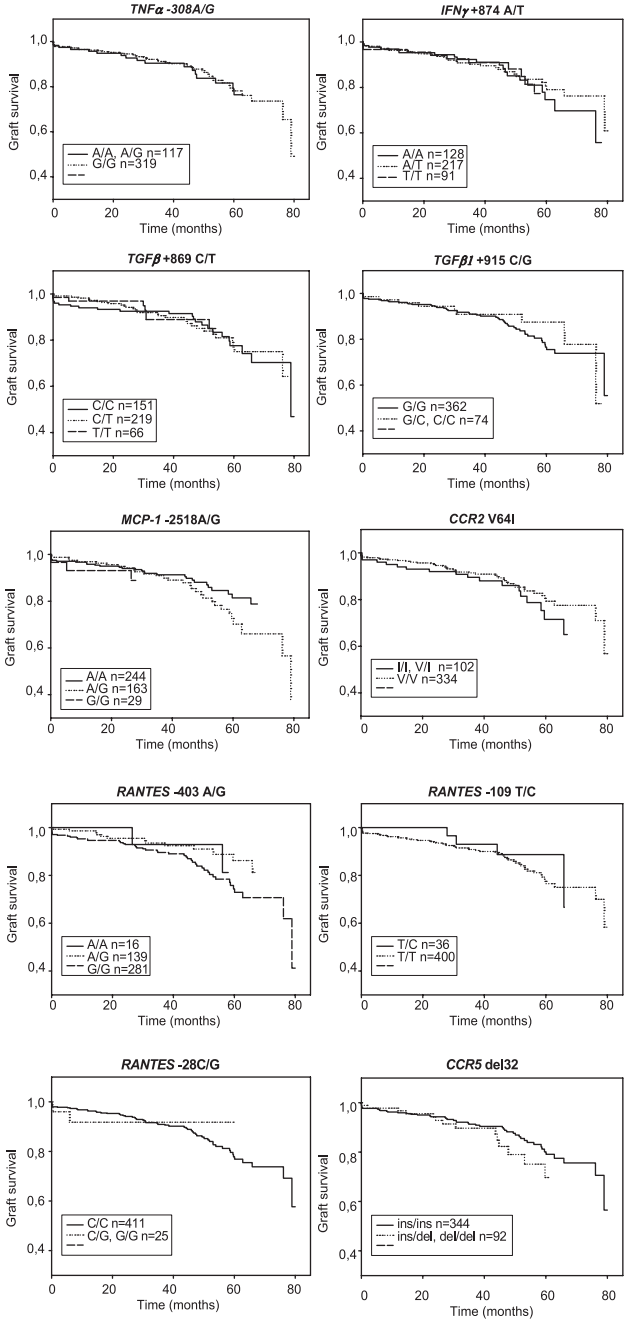

FIGURE 1. Influence of cytokine and chemokine gene polymorphisms on graft survival (n=436).

and *+915G/C* have been associated with an increased risk of systemic lupus erythematodes (*33*), psoriatic arthritis (*34*), human immunodeficiency virus resistance (*35*, *36*), tuberculosis (*14*), and progression of chronic renal insufficiency (*37*), supporting their role in the pathogenesis of human inflammatory disorders. In line with these reports, several groups found an association of the polymorphisms with acute or chronic kidney allograft rejection, with odds ratio for acute graft rejection ranging from 1.8 (*38*) to 10 (*16*) and for chronic allograft nephropathy ranging from 1.8 (*39*) to 3.1 (*22*).

In our study, we included only those cytokines whose role in the pathogenesis of renal allograft rejection was proved in animal models and those polymorphisms whose impact on the expression of the respective gene or the function on the respective protein was described in in vitro studies. However, in contrast to the published reports (*16*, *22*, *40*, *41*), we found no association between any of the functionally relevant polymorphisms and the risk of acute, subclinical rejection as well as chronic allograft nephropathy. There are several potential explanations for the discrepancy between our results and those of previous studies. These explanations refer to the currently accepted prerequisites for the design of genotype/phenotype association studies (*42*, *43*), to which the previous studies did not closely adhere.

At the outset, there should be a logical rationale for the chosen candidate genes and a coherent hypothesis based on the functional significance of the studied genetic variants. As regards the former, previous papers correctly studied cytokines involved in the Th1 and Th2-mediated pathogenesis of renal allograft rejection. However, in a study published by Sankaran et al. (*16*), individuals with hypersecretory phenotype of both TNF-α and interleukin (IL)-10 had an increased risk of acute allograft rejection, although IL-10 is an example of anti-inflammatory cytokine. In a transgenic mouse model, IL-10 inhibited production of TNF-α and neutrophil accumulation (*44*), which seems contradictory to the synergism of both cytokines postulated by Sankaran et al. (*16*).

Then, several functionally-related genes should be tested since this approach has a higher chance to detect genetic risk factors than the screening of single genetic variants. However, as an increasing number of comparisons increases the false-positive rate (*45*), an appropriate correction for multiple testing has to be implemented. Such correction would show that the association of the *TNF-*α $-$308A, *TGF-*β $+$915G and *IL-10* $-$1082G alleles with acute allograft rejection reported by Alakulppi et al. (*40*) is a flawed interpretation of the data.

Furthermore, subjects with comparable baseline characteristics should be included in order to eliminate possible confounding (*46*). In particular, the influence of genetic variability on rejection could be masked by immunosuppression even when present. For instance, Asderakis et al. (*22*) reported an increased risk for acute allograft rejection for carriers of the allele ^{*2} of the *IFN-*γ microsatellite polymorphism. The association was found only in the subgroup of 28 patients on cyclosporine monotherapy, but not in the whole cohort of 88 patients, of whom 60 received also steroids and azathioprine. It is likely that some of these individuals carrying the high producer *IFN-*γ^*2 allele might have developed rejection if they had been only on cyclosporine. The treatment heterogeneity caused an underestimation of patients with acute rejection and subsequent failure to assign them into the appropriate outcome group.

In addition, the reliability of the genotyping assays should be assured and the test for Hardy-Weinberg equilibrium (HWE) should be performed. Deviation from the HWE points at systematic genotyping error, which hampers any interpretation of the results, as for example in the study of Dmitrienko et al. (*41*) in which the low *P* value for HWE at the *TNF-*α $-$308 locus in the control group of healthy subjects indicates a significant genotyping inaccuracy.

Finally, the most obvious explanation for the discrepancy between our findings and previous studies is the lack of

statistical power in vast majority of them. The key determinant of quality in an association study is sample size (*43*), which should be determined by the power calculation in the study-designing phase. Results obtained from inadequately powered studies tend to have a decreased probability of detecting a true effect of a polymorphism due to the type II error (false negativity). Moreover, for any choice of significance level, the proportion of false-positive results among all positive results (type I error) is greatly increased as power decreases (*47*). Our calculation revealed that none of the studies reporting an association of polymorphisms in the proinflammatory genes with renal allograft rejection complied with the current demands for 80% power (*16*, *22*, *38*, *40*, *41*, *48*). Moreover, none of the studies included an independent validation cohort, which should be implemented, particularly in small studies that are likely to overestimate the true effect size (*49*).

In conclusion, none of the functionally relevant polymorphisms in the *TNF-α*, *MCP-1*, *RANTES*, *CCR2*, *CCR5*, *IFN-γ* and *TGF-β* genes increased the risk of acute, chronic, or subclinical renal allograft rejection in our cohort. Although there seems a little doubt that Th1- and Th2-mediated immune reactions play a role in the pathogenesis of renal allograft rejection, hereditary susceptibility to acute or chronic renal allograft rejection in white subjects has not been unequivocally proven. It remains possible that genetic predisposition to renal allograft rejection is determined by multiple polymorphisms with a low individual contribution to the phenotype that cannot be assessed in allelic association studies. Alternatively, other as yet unidentified polymorphisms may significantly affect the risk of renal allograft rejection. Therefore, identification of such polymorphisms warrants future studies that will be fully compliant with the currently accepted standards for polymorphism-disease association studies.

ACKNOWLEDGMENTS

We thank Alena Louzecka for excellent technical assistance, Michaela Prokopova for her help with clinical data collection, Věra Lánská for her help with statistical analysis, and Milan Jirsa for comments.

REFERENCES

1. Waller JR, Nicholson ML. Molecular mechanisms of renal allograft fibrosis. *Br J Surg* 2001; 88: 1429.
2. Baan CC, Weimar W. Intragraft cytokine gene expression: Implications for clinical transplantation. *Transpl Int* 1998; 11: 169.
3. Khan F, Agrawal S, Agrawal S. Genetic predisposition and renal allograft failure: Implication of non-HLA genetic variants. *Mol Diagn Ther* 2006; 10: 205.
4. Paul LC, Saito K, Davidoff A, Benediktsson H. Growth factor transcripts in rat renal transplants. *Am J Kidney Dis* 1996; 28: 441.
5. Nadeau KC, Azuma H, Tilney NL. Sequential cytokine dynamics in chronic rejection of rat renal allografts: Roles for cytokines RANTES and MCP-1. *Proc Natl Acad Sci U S A* 1995; 92: 8729.
6. Kitagawa K, Wada T, Furuichi K, et al. Blockade of CCR2 ameliorates progressive fibrosis in kidney. *Am J Pathol* 2004; 165: 237.
7. Eis V, Luckow B, Vielhauer V, et al. Chemokine receptor CCR1 but not CCR5 mediates leukocyte recruitment and subsequent renal fibrosis after unilateral ureteral obstruction. *J Am Soc Nephrol* 2004; 15: 337.
8. Sanderson N, Factor V, Nagy P, et al. Hepatic expression of mature transforming growth factor beta 1 in transgenic mice results in multiple tissue lesions. *Proc Natl Acad Sci U S A* 1995; 92: 2572.
9. Wilson AG, Symons JA, McDowell TL, et al. Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. *Proc Natl Acad Sci U S A* 1997; 94: 3195.
10. Rovin BH, Lu L, Saxena R. A novel polymorphism in the MCP-1 gene regulatory region that influences MCP-1 expression. *Biochem Biophys Res Commun* 1999; 259: 344.
11. Guignard F, Combadiere C, Tiffany HL, Murphy PM. Gene organization and promoter function for CC chemokine receptor 5 (CCR5). *J Immunol* 1998; 160: 985.
12. Nakayama EE, Tanaka Y, Nagai Y, et al. A CCR2–V64I polymorphism affects stability of CCR2A isoform. *AIDS* 2004; 18: 729.
13. Samson M, Libert F, Doranz BJ, et al. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* 1996; 382: 722.
14. Rossouw M, Nel HJ, Cooke GS, et al. Association between tuberculosis and a polymorphic NFkappaB binding site in the interferon gamma gene. *Lancet* 2003; 361: 1871.
15. Awad MR, El-Gamel A, Hasleton P, et al. Genotypic variation in the transforming growth factor-beta1 gene: Association with transforming growth factor-beta1 production, fibrotic lung disease, and graft fibrosis after lung transplantation. *Transplantation* 1998; 66: 1014.
16. Sankaran D, Asderakis A, Ashraf S, et al. Cytokine gene polymorphisms predict acute graft rejection following renal transplantation. *Kidney Int* 1999; 56: 281.
17. Pelletier R, Pravica V, Perrey C, et al. Evidence for a genetic predisposition towards acute rejection after kidney and simultaneous kidney-pancreas transplantation. *Transplantation* 2000; 70: 674.
18. Kruger B, Schroppel B, Ashkan R, et al. A Monocyte chemoattractant protein-1 (MCP-1) polymorphism and outcome after renal transplantation. *J Am Soc Nephrol* 2002; 13: 2585.
19. Abdi R, Tran TB, Sahagun-Ruiz A, et al. Chemokine receptor polymorphism and risk of acute rejection in human renal transplantation. *J Am Soc Nephrol* 2002; 13: 754.
20. Fischereder M, Luckow B, Hocher B, et al. CC chemokine receptor 5 and renal-transplant survival. *Lancet* 2001; 357: 1758.
21. McDaniel DO, Barber WH, Nguyan C, et al. Combined analysis of cytokine genotype polymorphism and the level of expression with allograft function in African-American renal transplant patients. *Transpl Immunol* 2003; 11: 107.
22. Asderakis A, Sankaran D, Dyer P, et al. Association of polymorphisms in the human interferon-gamma and interleukin-10 gene with acute and chronic kidney transplant outcome: The cytokine effect on transplantation. *Transplantation* 2001; 71: 674.
23. Racusen LC, Solez K, Colvin RB, et al. The Banff 97 working classification of renal allograft pathology. *Kidney Int* 1999; 55: 713.
24. Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. *Nephron* 1976; 16: 31.
25. Szalai C, Duba J, Prohaszka Z, et al. Involvement of polymorphisms in the chemokine system in the susceptibility for coronary artery disease (CAD). Coincidence of elevated Lp(a) and MCP-1-2518 G/G genotype in CAD patients. *Atherosclerosis* 2001; 158: 233.
26. Azzawi M, Pravica V, Hasleton PS, Hutchinson IV. A single nucleotide polymorphism at position −109 of the RANTES proximal promoter. *Eur J Immunogenet* 2001; 28: 95.
27. al Sharif F, Ollier WE, Hajeer AH. A rare polymorphism at position −28 in the human RANTES promoter. *Eur J Immunogenet* 1999; 26: 373.
28. Verjans GM, Brinkman BM, Van Doornik CE, et al. Polymorphism of tumour necrosis factor-alpha (TNF-alpha) at position −308 in relation to ankylosing spondylitis. *Clin Exp Immunol* 1994; 97: 45.
29. Pravica V, Perrey C, Stevens A, et al. A single nucleotide polymorphism in the first intron of the human IFN-gamma gene: Absolute correlation with a polymorphic CA microsatellite marker of high IFN-gamma production. *Hum Immunol* 2000; 61: 863.
30. Hubacek JA, Lacha J. Alternative method for diagnosis of two polymorphisms in the human transforming growth factor-beta1 by PCR-mediated double site-directed mutagenesis. *Clin Chim Acta* 2000; 295: 187.
31. The International HapMap Project. *Nature* 2003; 426: 789.
32. Morton NE. Linkage disequilibrium maps and association mapping. *J Clin Invest* 2005; 115: 1425.
33. Lee YH, Harley JB, Nath SK. Meta-analysis of TNF-alpha promoter −308 A/G polymorphism and SLE susceptibility. *Eur J Hum Genet* 2006; 14: 364.
34. Balding J, Kane D, Livingstone W, et al. Cytokine gene polymorphisms: association with psoriatic arthritis susceptibility and severity. *Arthritis Rheum* 2003; 48: 1408.
35. Modi WS, Goedert JJ, Strathdee S, et al. MCP-1-MCP-3-Eotaxin gene cluster influences HIV-1 transmission. *AIDS* 2003; 17: 2357.

36. Liu H, Chao D, Nakayama EE, et al. Polymorphism in RANTES chemokine promoter affects HIV-1 disease progression. *Proc Natl Acad Sci U S A* 1999; 96: 4581.
37. Khalil MS, El Nahas AM, Blakemore AI. Transforming growth factor-beta1 SNPs: genetic and phenotypic correlations in progressive kidney insufficiency. *Nephron Exp Nephrol* 2005; 101: e31.
38. Tinckam K, Rush D, Hutchinson I, et al. The relative importance of cytokine gene polymorphisms in the development of early and late acute rejection and six-month renal allograft pathology. *Transplantation* 2005; 79: 836.
39. Melk A, Henne T, Kollmar T, et al. Cytokine single nucleotide polymorphisms and intrarenal gene expression in chronic allograft nephropathy in children. *Kidney Int* 2003; 64: 314.
40. Alakulppi NS, Kyllonen LE, Jantti VT, et al. Cytokine gene polymorphisms and risks of acute rejection and delayed graft function after kidney transplantation. *Transplantation* 2004; 78: 1422.
41. Dmitrienko S, Hoar DI, Balshaw R, Keown PA. Immune response gene polymorphisms in renal transplant recipients. *Transplantation* 2005; 80: 1773.
42. Cooper DN, Nussbaum RL, Krawczak M. Proposed guidelines for papers describing DNA polymorphism-disease associations. *Hum Genet* 2002; 110: 207.
43. Hattersley AT, McCarthy MI. What makes a good genetic association study? *Lancet* 2005; 366: 1315.
44. Lee CG, Homer RJ, Cohn L, et al. Transgenic overexpression of interleukin (IL)-10 in the lung causes mucus metaplasia, tissue inflammation, and airway remodeling via IL-13-dependent and -independent pathways. *J Biol Chem* 2002; 277: 35466.
45. Altman DG MD, Bryant TN, Gardner MJ, eds. Statistics with Confidence BJM Books. BJM Books. Bristol, UK: Blackwell Publishing Ltd, 2000: 163.
46. Miettinen OS, Cook EF. Confounding: essence and detection. *Am J Epidemiol* 1981; 114: 593.
47. Kirkwood BSJ. Essential medical statistics. 2nd ed. Boston: Blackwell Science; 2003: 426.
48. Marshall SE, McLaren AJ, Haldar NA, et al. The impact of recipient cytokine genotype on acute rejection after renal transplantation. *Transplantation* 2000; 70: 1485.
49. Wacholder S, Chanock S, Garcia-Closas M, et al. Assessing the probability that a positive report is false: An approach for molecular epidemiology studies. *J Natl Cancer Inst* 2004; 96: 434.

PUBLIKAČNÍ AKTIVITY AUTORKY

I. PUBLIKACE

1. ***BRABCOVA I.***, TESAR V., HONSOVA E., LODEREROVA A., NOVOTNA E., MAIXNEROVA D., MERTA M., BURGELOVA M., HRIBOVA P., SKIBOVA J., ZADRAZIL J., MALY J., VIKLICKY O. Association of advanced vasculopathy and transforming growth factor-beta1 gene expression with immunoglobin A nephropathy progression. Nephrology Dialysis Transplantation,2010; doi10.1093/ndt/gfq423. IF 3,306

2. JACHYMOVA M., BRABCOVA I., GERMANOVA A., SLATINSKA J., MESTEK O., MATL I., ZIMA T., VIKLICKY O., KALOUSOVA M. A419C Polymorphism of Glyoxalase I Gene: Renal Function and Histological Findings at 12 Months after Renal Transplantation. Int J Nephrol Urol, 2010; 2(4): 504-513. IF 1,053

3. MATL I, HRIBOVA P, HONSOVA E, BRABCOVA I, VIKLICKY O. Potential predictive markers in protocol biopsies for premature renal graft loss. Kidney Blood Press Res. 2010;33(1):7-14. IF 1,714

4. KALOUSOVÁ M, BRABCOVÁ I, GERMANOVÁ A, JÁCHYMOVÁ M, MATL I, MESTEK O, BANDÚR S, ZIMA T, VIKLICKÝ O. RAGE polymorphisms, renal function and histological finding at 12 months after renal transplantation. Clin Biochem. 2009;42(4-5):347-52. IF 2,019

5. BANDUR S, PETRASEK J, HRIBOVA P, NOVOTNA E, BRABCOVA I, VIKLICKY O. Haplotypic structure of ABCB1/MDR1 gene modifies the risk of the acute allograft rejection in renal transplant recipients. Transplantation. 2008;86(9):1206-13. IF 3,972

6. HŘIBOVÁ, P., REINKE, P., PETRÁŠEK, J., BRABCOVÁ, I., HUBÁČEK, J., VIKLICKÝ, O. Heme oxygenase-1 polymorphisms and renal transplantation outcomes: balancing at the detection limit of allelic association studies. American journal of transplantation, 2008;8(5):1077-78. IF 6,843

7. ***BRABCOVÁ, I.***, PETRÁŠEK, J., HŘIBOVÁ, P., HYKLOVÁ, K., BARTOŠOVÁ, K., LÁCHA, J., VIKLICKÝ, O. Genetic variability of major inflammatory mediators has

no impact on the outcome of kidney transplantation. Transplantation, 2007;84(8):1037-44. IF 3,972

8. ***BRABCOVÁ, I***., KOTSCH, K., HŘIBOVÁ, P., LOUŽECKÁ, A., BARTOŠOVÁ, K., HYKLOVÁ, K., LÁCHA, J., VOLK, H., VIKLICKÝ, O. Intrarenal gene expression of proinflammatory chemokines and cytokines in chronic proteinuric glomerulopathies. Physiological Research, 2007;56(2):221-26. IF 2,093

9. SEILER, M., BRABCOVÁ, I., VIKLICKÝ, O., HŘIBOVÁ, P., ROSENBERGER, C., PRATSCHKE, J., LODERÉROVÁ, A., MATZ, M., SCHÖNEMANN, C., REINKE, P., VOLK, H. Heightened expression of the cytotoxicity receptor NKG2D correlates with acute and chronic nephropathy after kidney transplantation. American journal of transplantation, 2007;7(2):423-33. IF 6,843

10. HŘIBOVÁ, P., LÁCHA, J., KOTSCH, K., VOLK, H.D., BRABCOVÁ, I., SKIBOVÁ, J., VÍTKO, Š., VIKLICKÝ, O. Intrarenal cytokine and chemokine gene expression and kidney graft outcome. Kidney and Blood Pressure Research, 2007;30(5):273-82. IF 1,895

11. HŘIBOVÁ, P., KOTCH, K., BRABCOVÁ, I., VÍTKO Š, VOLK, H.D., LACHA, J. Cytokines and chemokine gene expression in human kidney transplantation. Transplant. Proc., 2005;37(2):760-63. 0,799

12. LÁCHA, J., HŘIBOVÁ, P., KOTCH, K., BRABCOVÁ, I., BARTOŠOVÁ, K., VOLK, H.D., VÍTKO, Š. Effect of cytokines and chemokines (TGF-beta, TNF-alpha, IL-6, IL-10, MCP-1, RANTES) gene polymorphisms in kidney recipients on posttransplantation outcome: influence of donor-recipient match. Transplant Proc., 2005;37(2):764-6. IF 0,799

13. ***BRABCOVÁ, I***., VIKLICKÝ, O., LÁCHA, J. Úloha chemokinů při rejekci transplantovaných orgánů. Alergie, 2005, roč. 7, č. 4, str. 269-273.

Celkem reference autorky:

Celkem IF: 35,308

H-index: 5

SCI: 72

PREZENTACE NA KONGRESECH A SYMPOZIÍCH

i. ZAHRANIČNÍ

14. ***Brabcova I***, Novotna E, Lodererova A, Honsova E, Skibova J, Burgelova M, Merta M, Maixnerova D, Rysava R, Tesar V and Viklický O. Gene expression profiles and mononuclear cell infiltration in IgA nephropathy and focal and segmental glomerulopathy, World Congress of Nephrology 2009

15. ***Brabcova I***, Novotna E, Lodererova A, Honsova E, Skibova J, Burgelova M, Merta M, Maixnerova D, Rysava R, Tesar V and Viklický O. Gene expression profiles and mononuclear cell infiltration in IgA nephropathy and focal and segmental glomerulopathy, IgA Nephropathy - WCN 2009 Satellite Symposium 2009

16. ***Brabcova I***, Lodererova A, Maixnerova D, Tesar V, Merta M, Burgelova M, Honsova E, Novotna E, Rysava R, Zadrazil J a Viklicky O. Gene expression patterns and interstitial infiltration in IgAN and FSGS. Young investigator forum 2009

17. ***Brabcova I***, Merta M, Maixnerova D, Hribova P, Novotna E, Tesar V, Rysava R, Lodererova A, Honsova E and Viklicky O. Cytokine and chemokine gene expression and mononuclear cell infiltration in IgA nephropathy. XLV European Renal Association – European Dialysis Transplant Association Congress 2008

18. ***Brabcova I***, Petrasek J, Hribova P, Hyklova K, Bartosova K, Lacha J, Viklicky O. Cytokine and chemokine gene variants and kidney graft outcome. Transplant International, 13th Congress of the European Society for Organ Transplantation 2007

19. Matl I, Viklicky O, Lyerova L, Honsova E, Hribova P, Brabcova I, Lanska V. Risk factors of subclinical acute rejection in 3-months protocol kidney biopsy. 13th Congress of the European Society for Organ Transplantation 2007

20. Klippert K, Hribova P, Brabcova I, Slavcev A, Bartosova K, Viklicky O, Kunert K, Volk HD, Kotsch K. Monitoring of killer-cell immunoglobulin-like receptor (KIR)

expression following renal transplantation. 13th Congress of the European Society for Organ Transplantation 2007

21. Hribova P., Kotsch K., Brabcova I., Volk H., Vitko S., Viklicky O. Renal allograft nephropathy and intrarenal cytokine and chemokine gene expression. World Transplant Congress of Nephrology 2007

22. Klippert K, Hribova P, Brabcova I, Slavcev A, Kunert K, Volk HD, Viklicky O, Kotsch K. Protein and mRNA expression of killer-cell immunoglobulin-like receptors (KIR) in peripheral blood following renal transplantation. American Transplant Congress 2007

23. Hribova P, Brabcova I, Kotsch K, Volk H-D, Skibova J, Lacha J, Viklicky O. Intrarenal cytokine and chemokine gene expression in chronic allograft nephropathy. World Congress of Nephrology 2007

24. ***Brabcova I***, Hribova P, Hyklova K, Bartosova K, Matl I, Viklicky O. IL-10 –1082 gene variants influence kidney graft function at 12 months. World Transplant Congress 2006

25. Hribova P, Brabcova I, Kotsch K, Volk H-D, Skibová J, Lacha J, Viklicky O. Intrarenal cytokine and chemokine gene expression in chronic allograft nephropathy. World Transplant Congress 2006

26. Hribova P, Bartosova K, Slatinska J, Hyklova K, Brabcova I, Volk HD, Viklicky O. Higher intrarenal TGF-β1 gene expression in early acute humoral rejection of renal allografts. World Transplant Congress 2006

27. Viklicky O, Brabcova I, Hubacek JA, Hribova P, Slavcev A, Hyklova K, Volk HD, Vitko S, Reinke P. Recipient promoter variant T(-413)A of the heme oxygenase-1 gene influences kidney allograft survival and acute rejection incidence. World Transplant Congress 2006

28. Hribova P, Brabcova I, Kotsch K, Volk HD, Skibova J, Lacha J, Viklicky O. Intragraft cytokine and chemokine gene expression in chronic allograft nephropathy influences graft failure in the long-term. ESH euroconference on translational research in transplantation: immunogenetics, pharmacogenomics, proteomics and immunobiology 2006

29. Lácha J, Hřibová P, Kotsch K, Brabcová I, Lodererová A, Honsová E, Volk H-D. TGF-β mRNA up-regulation predicts chronic renal allograft dysfunction within 18 months follow-up independently from the initial histological diagnosis. American Transplan Congress 2005

30. Hribova P, Kotsch K, Brabcova I, Vitko S, Volk H-D, Lacha J. Cytokines and chemokines gene expression in human kidney transplants. XX international congress of the transplantaion society 2004

31. Lacha J, Hribova P, Kotsch K, Brabcova I, Bartosova K, Volk H-D, Vitko S. The effect of cytokines and chemokines (TGF-β, TNF-α, IL-6, IL-10, MCP-1, RANTES, CD-14) gene polymorphisms in kidney recipients on post transplant outcome: the influence of donor-recipients match. XX international congress of the transplantaion society 2004

ii. ČESKÉ

1. Hřibová P., Brabcová I., Slatinská J., Honsová E., Zachoval R., Viklický O. Genetická heterogenita chronické rejekce transplantované ledviny. III. Československý transplantační kongres 2010

2. ***Brabcova I***, Hribova P, Hyklova K, Lanska V, Bartosova K, Lacha J, Viklicky O. Vliv polymorfizmů v genech pro cytokiny, chemokiny a chemokinové receptory na osud transplantované ledviny. I. Československý transplantační kongres 2006

3. Viklicky O, Brabcova I, Hubacek JA, Hribova P, Slavcev A, Hyklova K, Vitko S. Polymorfizmus genu pro hemoxygenázu 1 ovlivňuje výskyt akutních rejekcí a přežití transplantovaných ledvin. I. Československý transplantační kongres 2006

4. Hribova P, Lacha J, Volk H-D, Kotsch K, Skibova J, Brabcova I, Vitko S, Viklicky O. Zvýšená intrarenální exprese cytokinů a chemokinů: riziko selhání transplantované ledviny. I. Československý transplantační kongres 2006

5. Hyklova K, Hribova P, Brabcova I, Viklicky O. Molekulárně genetické metody a jejich využití v transplantační medicíně. I. Československý transplantační kongres 2006

6. Hubacek JA, Bohuslavova R, Brabcova I, Hribova P, Volk H-D, Viklicky O. Polymorfismus v regulační oblasti genu pro hemoxygenázu-1 jako rizikový faktor renálního selhání. I. Československý transplantační kongres 2006

7. ***Brabcova I***, Hribova P, Hyklova K, Bartosova K, Matl I, Viklicky O. Vliv IL-10-1082A/G polymorfismu na funkci renálního štěpu ve 12.měsíci po transplantaci. 31. kongres České nefrologické společnosti 2006

8. Viklicky O, Hubacek J, Brabcova I, Hribova P, Bartosova K, Vitko S, Volk HD . Polymorfizmus T(-413)A genu pro hemoxygenázu 1 ovlivňuje výskyt akutních rejekcí a přežití transplantovaných ledvin. 31. kongres České nefrologické společnosti 2006

9. ***Brabcova I***, Hribova P, Kotsch K, Chalupnikova K, Louzecka A, Volk H-D, Lacha J. Vliv polymorfismu chemokinů a chemokinových receptorů na osud transplantovaného orgánu. 6. kongres České transplantační společnosti 2004

10. Hribova P, Kotsch K, Brabcova I, Vitko S, Volk H-D, Lacha J. Exprese cytokinů a chemokinů v lidské transplantované ledvině. Aktuality v nefrologii 2004; 10(Suppl 1): 27; 6. kongres České transplantační společnosti 2004

11. Lacha J, Hribova P, Kotsch K, Brabcova I, Bartosova K, Volk H-D, Hubacek J, Vitko S. Vliv polymorfismu genů pro cytokiny a chemokiny (IL-6, IL-10, TGF-β1, TNF-α, MCP-1, RANTES, CD14) u dárců a příjemců na funkci ledvinného štěpu. 6. kongres České transplantační společnosti 2004