Risk of cutaneous squamous cell carcinoma development in renal transplant recipients is independent of *TMC/EVER* alterations

Running head: *TMC* alterations and risk of SCC development in RTR

Topic: Clinical and Laboratory Studies

Number of words: 2133

Number of tables and figures: 2 tables and 0 figures

B. Burger[1], I. Spoerri[1], D. A. Stegmann[1], J. De Mesmaker[1], S. Schaub [2], P. H. Itin[3], J. Steiger[4], A. W. Arnold[3]

[1] Research Group of Dermatology, Department of Biomedicine, University Hospital Basel and University of Basel, Basel, Switzerland

[2] Clinic for Transplantation Immunology and Nephrology, University Hospital Basel, Basel, Switzerland

[3] Clinic for Dermatology, University Hospital Basel, Basel, Switzerland

[4] Division of Transplant Immunology & Nephrology, University Hospital Basel, Basel, Switzerland

Corresponding author:

Bettina Burger, PhD

Department of Biomedicine, Hebelstr. 20, 4031 Basel, Switzerland

phone: ++41 61 328 69 03, fax: ++41 61 265 23 50, e-mail: bettina.burger@usb.ch

Key words

Renal transplant recipients, squamous cell carcinoma, TMC, EVER, single nucleotide polymorphism

Disclosure

All authors declared no competing interests or financial interest.

Funding

The study was supported by “Fondation Claude et Giuliana”

Abstract

Background: Renal-transplant recipients (RTR) have an increased risk of developing non-melanoma-skin-cancer, mainly squamous cell carcinoma (cSCC). Two genes (TMC/EVER), mutated in epidermodysplasia verruciformis patients (EV) with an increased risk of cSCC development, contain numerous single-nucleotide-polymorphisms (SNP).

Aim: To evaluate the effect of SNPs in both TMC/EVER genes on different susceptibility of RTRs to cSCC.

Method: We determined the occurrence of cSCC in 105 RTR who were transplanted at least 7 years ago and investigated the frequency of 26 SNPs within both TMC/EVER genes in severely-affected (n=16) as well as in not-affected RTR (n=25).

Results: Our data did not indicate a significant association between any SNP genotype and risk of cSCC development in RTR.

Conclusion: To clarify the correlation between SNPs in TMC and cSCC development in RTR, integrated investigations of large cohorts including both RTR and immunocompetent individuals with consideration of cSCC status, SNP genotype, and HPV status might be necessary.

Introduction

Kidney transplantation is the preferred modality of renal replacement therapy for many patient with end stage renal disease. As short-term patient and allograft survival are excellent nowadays, improvement of long-term morbidity and mortality due to malignancies have emerged as key goals. Renal transplant recipients (RTR) have at least a 3 to 4-fold increased risk of developing cutaneous cancer after transplantation compared to the general population [1]. The most common cancer in RTR is non-melanoma skin cancer (NMSC). While the usual incidence ratio of SCC:BCC is 1:4 in the immunocompetent population, this ratio is reversed in transplant recipients. The risk of developing cutaneous squamous cell cancer (cSCC) is estimated to be 65-fold higher than for the general population [2]. Therefore more than 36% of all transplanted and immunosuppressed patients develop at least one NMSC after transplantation [3,4]. UV irradiation, type and duration of immunosuppression, as well as age at transplantation and time period after transplantation consist the main risk factors for the development of cSCCs. Furthermore, fair skin as well as history of prior skin cancer and actinic keratoses are known to increase the risk of NMSC emergence [5,6] but do not cover the individual risk. Therefore all RTR are yearly supplied with follow-up examinations. Identification of a genetic risk factor might help to estimate the individual risk and to establish an individual monitoring resulting in lower healthcare costs.

Epidermodysplasia verruciformis (EV) is a rare autosomal recessive disorder leading to an increased susceptibility to persistent infections by cutaneous β-human papillomaviruses (β-HPV) [7]. Patients with EV develop disseminated cutaneous wart-like lesions and have an increased risk of developing cSCC in sun exposed skin areas, which is comparable to the risk of skin cancer development in RTR. In 2002, homozygous nonsense and frameshift mutations in the *TMC6/EVER1* and *TMC6/EVER2* genes have been identified in patients with EV [8] and could be confirmed in about 75% of patients. These loss-of-function mutations result in an increased susceptibility to infections by specific HPVs of the genus β, mainly HPV5 and 8 [8]. Both *TMC/EVER* genes contain numerous single nucleotide polymorphisms (SNP), most of them leading to missense mutations. Their relevance on cancer development has been discussed recently [9-11].

Likewise another SNP (rs1042522) in the *TP53* gene (c. 215CCC>CGC; p.Pro72Arg) at codon 72 has been controversially discussed to be associated with cancer development [12-16]. This alteration possibly increases the susceptibility of the TP53 protein to degradation mediated by E6 of HPVs [17,18]. An important hint for the involvement of SNP rs1042522 in non-melanoma skin cancer development is its homozygosity in a considerable number of EV patients [19]. Furthermore this SNP was significantly associated with NMSC development in RTRs [20].

The objective of our pilot study was to evaluate a direct correlation of SNPs within *TMC6/EVER1* and *TMC8/EVER2* as well as to prove influences of rs1042522 in *TP53* on an increased risk of cSCC development in RTR.

Materials and Methods

Study population

The study was approved by the Ethics Committee of Basel, Switzerland (EK11/10) and informed consent was given by all participants. The procedures were in accordance with the Helsinki Declaration.

105 RTR of Central European origin who had been transplanted and followed after transplantation at one center (University Hospital Basel, Switzerland) at least 7 years ago were included in the study; data were collected from patient records. Patients were classified according to the number of previous and current NMSC and warts as well as type of medically induced immunosuppression, Fitzpatrick skin type, and UV exposition. Patients with a history of cancer other than cSCC or basal cell carcinoma (BCC) were not included in the study. Patients were grouped into severely affected (multiple (≥3) cSCC, group 1, n=16), moderately affected (development of one or two cSCC or no cSCC but BCC and/or precancerous lesions, group 2, n=64), and not-affected patients (no cSCC, no warts, no precancerous lesions, group 3, n=25). The anonymous control group (n = 113) included non-transplanted individuals from the general Central European population without known increased risk of cSCC. The mean age at transplantation was 42 years (group1), 46 years (group 2) and 40 years (group 3). The male:female ratio was 4:1 (group 1), 1.3:1 (group 2), and 1.4:1 (group 3).

All patients have received immunosuppressive treatment with cyclosporine, azathioprine, prednisone, rapamycin, mycophenolatmophetil, and tacrolimus. On most of them the drugs were administered in combined therapy (e.g. cyclosporine/prednisone, azathioprine/prednisone, cyclosporine/mycophenolatemophetil, rapamycin/mycophenolatemophetil). Comparison of medication between the patients showed a comparable immunosuppressive treatment for all groups. In only one patient immunosuppressive treatment was stopped because of severe progression of cancers with brain invasion and skin metastasis.

DNA preparation and SNP analysis

Genomic DNA was extracted from peripheral blood lymphocytes following standard salting out procedure. PCR (Qiagen, Valencia, CA, USA) was performed by means of primers amplifying the entire coding sequences of the *TMC6* and *TMC8* genes as well as exon intron boundaries. Primer

sequences were kindly imparted by G. Orth, France. In order to test our hypothesis in a pilot study, we focused on comparing genotypes of RTR, who were severely affected by multiple cSCCs and precancerous lesions (group 1) and patients not affected by cSCC, precancerous lesions or warts (group 3). In a first attempt to detect SNPs influencing cSCC development in RTRs purified PCR products (NucleoSpin Extract II, Machery-Nagel) of individuals in group 1 (n = 5) and group 3 (n = 4) were subjected to bi-directional sequencing using the Big-Dye terminator kit (v 3.1) and ABI Prism 377 sequencer (Applied Biosystems, Foster City, CA). Distinguished non-synonymous SNPs or SNPs possibly affecting the splice sites were screened in all RTR patients of both groups. Screening of *TMC/EVER* SNPs was carried out either by TaqMan® SNP Genotyping Assay (Applied Biosystems, Carlsbad, CA) according to the manufacturers instruction or by restriction fragment length polymorphism analysis (RFLP). For RFLP, PCR products spanning the region of interest were digested with site-specific enzyme (New England Biolabs) according to manufacturer's instructions (Table 1) (primer sequences are available on request). *TP53* SNP rs1042522 was characterized by RFLP after digestion of the PCR product with FauI (New England Biolabs).

Statistical Analysis

Data analysis of the genotypes was assessed using the statistical program R (http://www.r-project.org/). In case if the presence of three different genotypes for a specific SNP the significance was calculated by the Cochran-Armitage test (CATT) and logistic regression. In case of only two different genotypes for a specific SNP the Fisher test (FT) was applied. All significances were calculated using the additive model.

Results

In our study collective 16 patients (15.2%) were severely affected by cSCC (group 1). Patients in this group (n = 10/62.5%) suffered from multiple cSCC as well as from multiple precancerous lesions, which developed between 3 and 24 years after transplantation (median age at first cSCC 58$\pm$14 years). Four patients (25%) were afflicted with 4 to 8 cSCC additional to precancerous lesions and two patients (12.5%) developed three cSCC combined with multiple precancerous lesions at an early time point after transplantation (6 and 9 years). Group 2 consisted of 64 patients (61.0%), who developed only one, two, or no cSCC but one precancerous lesion or BCC at least. Group 3 consisted of 25 patients (23.8%) who have never developed cSCC, BCC, or precancerous lesions (median age at examination 54$\pm$12 years).

Genotypes in both *TMC/EVER* genes were investigated in patient groups, which differed most strongly regarding development of cSCC (group 1 and 3). Sequence analysis of all exons as well as

of exon-intron boundaries of both *TMC/EVER* genes revealed 23 SNPs (*TMC6/EVER1*) and 27 SNPs (*TMC8/EVER2*) present heterozygously or homozygously. One is a novel variant (c.1766G>A, p.(Arg586His)) in the exon 14 of *TMC8/EVER2*. Comparison of the frequency of 26 selected SNPs revealed no significant difference between group 1 and group 3 as well as compared to the control group (Table 2). Notably, no significant discrepancy was found for rs7208422 (*TMC8* c.917A>T, p.(Asn306Ile); p=0.15) among the investigated groups. The novel SNP in *TMC8* (c.1766G>A) showed a minor allele frequency (MAF) of A = 0.020/1 in all RTR patients. Overall, the MAF in both RTR groups as well as in the control group ranged from 0.014 (rs16970849) to 0.049 (rs7208422).

The allele frequency of rs1042522 in *TP53* did not significantly differ between the RTR groups (group 1 MAF: C=0.219/7, group 3 MAF: C=0.220/11) or in comparison to the control group (MAF: C=0.258/51) (Table 1).

Discussion

Influences of SNPs on cancer development have been debated since their discovery [21,22]. TMC6/EVER1 and TMC8/EVER2 are known to be involved in the development of EV. Homozygous deleterious alterations in either gene promote the susceptibility of the mutant carrier to β-HPV and cSCC [23]. Beta-HPV are discussed to have an impact on cSCC development in RTRs [24-26], implicating that SNPs in *TMC6/EVER1* and *TMC8/EVER2* might consist a risk factor for the different susceptibility of RTRs to cSCC. To test this hypothesis we examined RTR starting from seven to 42 years after transplantation for SNPs in both *TMC/EVER* genes. Number of cSCC, age of onset, and sex ratio of our study cohort are comparable to previously published data [2,6,27,28].

Our investigation of *TMC6/EVER1* and *TMC8/EVER2* in RTR revealed 26 different SNPs causing missense or silent mutations or being located in the introns near the exon-intron boundaries. We compared the frequency of these SNPs between RTR with severe cSCC affection and RTR without any skin cancer. Surprisingly, statistical analyses by the CATT or FT could not identify any SNP significantly associated with an increased risk of the development of cSCC in RTR. Neither risk analysis by logistic regression revealed any statistical significance.

Influences of SNPs on both *TMC/EVER* genes on cSCC development have been investigated recently. First studies examined correlation of a SNP in *TMC8/EVER2* (rs7208422), which is located in exon 8 of *TMC8* and leads to a missense mutation (c.917A>T, p.(Ile306Asp)). This SNP causes a reduced binding activity of TMC8 to TRADD (TNFRSF1A-associated via death domain) and leads to a less efficient apoptosis activation compared to the wildtype variant [29]. Its variant T was homozygously detected in a young HIV positive patient with acquired EV, whereas the HIV posi-

tive mother was heterozygously detected for the SNP and lacked EV lesions [30]. Two sisters with classical EV but without nonsense or frameshift mutation in either of the *TMC* gene were also homozygous for the more rare SNP variant T [31]. Therefore we hypothesized this SNP to influence the individual risk of cSCC development in combination with immunosuppression. In the non-EV population little information is known about the correlation of rs7208422, cSCC development, and HPV infection. One study investigating this SNP in the general population reported a slightly increased OR (1.7) for individuals with the homozygous T genotype compared to the homozygous A genotype [32], a trend we could not confirm in RTR. Our data does not support any significant influence of rs7208422 on development of cSCC in the investigated RTR collective. Interestingly, among RTR more than 82% of the cSCCs are HPV positive, which is in contrast to the general population with a HPV DNA prevalence of only 27% in cSCC [33]. Furthermore, within a transplanted but cSCC negative control group more than 60% of carriers of the homozygous variant A (rs7208422) or variant G (rs12452890) disclosed a significant association of seropositivity with β2-HPVs [34]. In compliance with our data no association was recently found between 11 specific *TMC/EVER* SNPs and cSCC development in RTRs and cardiac transplant recipients [34]. Of note, we have investigated 22 further *TMC* SNPs in our study, which also do not show any significant correlation to cSCC development among RTR. This includes a SNP in *TMC6* (rs12449858) which has been detected in a family with EV and was suggested to be correlated with EV development [35].

Examination of a further SNP (rs1042522 in the *TP53* gene), suggested to be involved in cSCC development [20], showed no correlation with cSCC risk in RTR, which is consistent with a single report [36] and a recently published meta-analysis [37].

Our pilot study is limited by an unequal distribution of male:female ratio as well as a missing HPV analysis of the cSCC. Additionally, it was impossible to evaluate patients' age at onset of the first cSCC because of missing data in the patient record. The presented survey examined few patients but all of them were renal transplanted. In correlation with other studies on transplanted patients we could not detect any correlation between *TMC/EVER* SNPs and increased risk of cSCC development. Possibly, an effect of *TMC/EVER* SNPs to cSCC development is hidden by other important influences e.g. the type of HPV infection, age, sex, or skin type. Combined studies on large cohorts of the general as well as the RTR population, investigating SNP genotype as well as HPV infection and cSCC status, might be necessary to detect a possible influence of *TMC/EVER* SNPs on HPV susceptibility and cSCC development.

Acknowledgement

We thank all patients for their participation as well as Robert Ivanek who supported the statistical analyses.

References

1. Lindelof B, Sigurgeirsson B, Gabel H, Stern RS: Incidence of skin cancer in 5356 patients following organ transplantation. Br J Dermatol 2000;143:513-519.

2. Jensen P, Hansen S, Moller B, Leivestad T, Pfeffer P, Geiran O, Fauchald P, Simonsen S: Skin cancer in kidney and heart transplant recipients and different long-term immunosuppressive therapy regimens. J Am Acad Dermatol 1999;40:177-186.

3. Gallagher MP, Kelly PJ, Jardine M, Perkovic V, Cass A, Craig JC, Eris J, Webster AC: Long-term cancer risk of immunosuppressive regimens after kidney transplantation. J Am Soc Nephrol 2010;21:852-858.

4. Kovach BT, Stasko T: Skin cancer after transplantation. Transplant Rev (Orlando) 2009;23:178-189.

5. Tessari G, Naldi L, Boschiero L, Minetti E, Sandrini S, Nacchia F, Valerio F, Rugiu C, Sassi F, Gotti E, Fonte L, Talamini G, Girolomoni G: Incidence of primary and second cancers in renal transplant recipients: A multicenter cohort study. Am J Transplant 2013;13:214-221.

6. Bouwes Bavinck JN, Euvrard S, Naldi L, Nindl I, Proby CM, Neale R, Abeni D, Tessari GP, Feltkamp MC, Claudy A, Stockfleth E, Harwood CA, group E-H-U-C: Keratotic skin lesions and other risk factors are associated with skin cancer in organ-transplant recipients: A case-control study in the netherlands, united kingdom, germany, france, and italy. J Invest Dermatol 2007;127:1647-1656.

7. Burger B, Itin PH: Epidermodysplasia verruciformis. Curr Probl Dermatol 2014;45:123-131.

8. Ramoz N, Rueda LA, Bouadjar B, Montoya LS, Orth G, Favre M: Mutations in two adjacent novel genes are associated with epidermodysplasia verruciformis. Nat Genet 2002;32:579-581.

9. Castro FA, Ivansson EL, Schmitt M, Juko-Pecirep I, Kjellberg L, Hildesheim A, Gyllensten UB, Pawlita M: Contribution of tmc6 and tmc8 (ever1 and ever2) variants to cervical cancer susceptibility. Int J Cancer 2011

10. Wang SS, Gonzalez P, Yu K, Porras C, Li Q, Safaeian M, Rodriguez AC, Sherman ME, Bratti C, Schiffman M, Wacholder S, Burk RD, Herrero R, Chanock SJ, Hildesheim A: Common genetic variants and risk for hpv persistence and progression to cervical cancer. PLoS One 2010;5:e8667.

11. Johanneson B, Chen D, Enroth S, Cui T, Gyllensten U: Systematic validation of hypothesis-driven candidate genes for cervical cancer in a genome-wide association study. Carcinogenesis 2014;35:2084-2088.

12. Cotignola J, Chou JF, Roy P, Mitra N, Busam K, Halpern AC, Orlow I: Investigation of the effect of mdm2 snp309 and tp53 arg72pro polymorphisms on the age of onset of cutaneous melanoma. J Invest Dermatol 2012;132:1471-1478.

13. Harris N, Brill E, Shohat O, Prokocimer M, Wolf D, Arai N, Rotter V: Molecular basis for heterogeneity of the human p53 protein. Mol Cell Biol 1986;6:4650-4656.

14. Matlashewski GJ, Tuck S, Pim D, Lamb P, Schneider J, Crawford LV: Primary structure polymorphism at amino acid residue 72 of human p53. Mol Cell Biol 1987;7:961-963.

15. Cheng C, Lingyan W, Yi H, Cheng Z, Huadan Y, Xuting X, Leiting X, Meng Y, Shiwei D: Association between tlr2, mtr, mtrr, xpc, tp73, tp53 genetic polymorphisms and gastric cancer: A meta-analysis. Clin Res Hepatol Gastroenterol 2014;38:346-359.

16. Marin MC, Jost CA, Brooks LA, Irwin MS, O'Nions J, Tidy JA, James N, McGregor JM, Harwood CA, Yulug IG, Vousden KH, Allday MJ, Gusterson B, Ikawa S, Hinds PW, Crook T, Kaelin WG, Jr.: A common polymorphism acts as an intragenic modifier of mutant p53 behaviour. Nat Genet 2000;25:47-54.

17. Storey A, Thomas M, Kalita A, Harwood C, Gardiol D, Mantovani F, Breuer J, Leigh IM, Matlashewski G, Banks L: Role of a p53 polymorphism in the development of human papillomavirus-associated cancer. Nature 1998;393:229-234.

18. Yang J, Liu B, Li W, Xiong H, Qiu H, Fu Q, Chen B, Hu G, Yuan X: Association of p53 and mdm2 polymorphisms with risk of human papillomavirus (hpv)-related esophageal squamous cell carcinoma (escc). Cancer Epidemiol 2013;37:629-633.

19. de Oliveira WR, Rady PL, Grady J, Hughes TK, Neto CF, Rivitti EA, Tyring SK: Association of p53 arginine polymorphism with skin cancer. Int J Dermatol 2004;43:489-493.

20. McGregor JM, Harwood CA, Brooks L, Fisher SA, Kelly DA, O'Nions J, Young AR, Surentheran T, Breuer J, Millard TP, Lewis CM, Leigh IM, Storey A, Crook T: Relationship between p53 codon 72 polymorphism and susceptibility to sunburn and skin cancer. J Invest Dermatol 2002;119:84-90.

21. Shastry BS: Snps: Impact on gene function and phenotype. Methods Mol Biol 2009;578:3-22.

22. Venza M, Catalano T, Visalli M, Venza I, Lentini M, Curia MC, Mariani-Costantini R, Teti D: Association of the dss1 c.143g>a polymorphism with skin squamous cell carcinoma. J Invest Dermatol 2010

23. Lazarczyk M, Cassonnet P, Pons C, Jacob Y, Favre M: The ever proteins as a natural barrier against papillomaviruses: A new insight into the pathogenesis of human papillomavirus infections. Microbiol Mol Biol Rev 2009;73:348-370.

24. Proby CM, Harwood CA, Neale RE, Green AC, Euvrard S, Naldi L, Tessari G, Feltkamp MC, de Koning MN, Quint WG, Waterboer T, Pawlita M, Weissenborn S, Wieland U, Pfister H, Stockfleth E, Nindl I, Abeni D, Schegget JT, Bouwes Bavinck JN: A case-control study of

betapapillomavirus infection and cutaneous squamous cell carcinoma in organ transplant recipients. Am J Transplant 2011;11:1498-1508.

25. Purdie KJ, Surentheran T, Sterling JC, Bell L, McGregor JM, Proby CM, Harwood CA, Breuer J: Human papillomavirus gene expression in cutaneous squamous cell carcinomas from immunosuppressed and immunocompetent individuals. J Invest Dermatol 2005;125:98-107.

26. Karagas MR, Waterboer T, Li Z, Nelson HH, Michael KM, Bavinck JN, Perry AE, Spencer SK, Daling J, Green AC, Pawlita M: Genus beta human papillomaviruses and incidence of basal cell and squamous cell carcinomas of skin: Population based case-control study. Bmj 2010;341:c2986.

27. Keller B, Braathen LR, Marti HP, Hunger RE: Skin cancers in renal transplant recipients: A description of the renal transplant cohort in bern. Swiss Med Wkly 2010;140:w13036.

28. Berg D, Otley CC: Skin cancer in organ transplant recipients: Epidemiology, pathogenesis, and management. J Am Acad Dermatol 2002;47:1-17; quiz 18-20.

29. Gaud G, Guillemot D, Jacob Y, Favre M, Vuillier F: Ever2 protein binds tradd to promote tnf-alpha-induced apoptosis. Cell Death Dis 2013;4:e499.

30. Hohenstein E, Rady PL, Hergersberg M, Huber AR, Tyring SK, Bregenzer T, Streit M, Itin P: Epidermodysplasia verruciformis in a hiv-positive patient homozygous for the c917a-->t polymorphism in the tmc8/ever2 gene. Dermatology 2009;218:114-118.

31. Arnold AW, Burger B, Kump E, Rufle A, Tyring SK, Kempf W, Hausermann P, Itin PH: Homozygosity for the c.917a --> t (p.N306l) polymorphism in the ever2/tmc8 gene of two sisters with epidermodysplasia verruciformis lewandowsky-lutz originally described by wilhelm lutz. Dermatology 2010

32. Patel AS, Karagas MR, Pawlita M, Waterboer T, Nelson HH: Cutaneous human papillomavirus infection, the ever2 gene and incidence of squamous cell carcinoma: A case-control study. Int J Cancer 2008;122:2377-2379.

33. Harwood CA, Surentheran T, McGregor JM, Spink PJ, Leigh IM, Breuer J, Proby CM: Human papillomavirus infection and non-melanoma skin cancer in immunosuppressed and immunocompetent individuals. J Med Virol 2000;61:289-297.

34. Madeleine MM, Carter JJ, Johnson LG, Wipf GC, Davis C, Berg D, Nelson K, Daling JR, Schwartz SM, Galloway DA: Risk of squamous cell skin cancer after organ transplant associated with antibodies to cutaneous papillomaviruses, polyomaviruses, and tmc6/8 (ever1/2) variants. Cancer Med 2014;3:1440-1447.

35. Zuo YG, Ma D, Zhang Y, Qiao J, Wang B: Identification of a novel mutation and a genetic polymorphism of ever1 gene in two families with epidermodysplasia verruciformis. J Dermatol Sci 2006;44:153-159.

36. O'Connor DP, Kay EW, Leader M, Atkins GJ, Murphy GM, Mabruk MJ: P53 codon 72 polymorphism and human papillomavirus associated skin cancer. J Clin Pathol 2001;54:539-542.

37. Liu T, Lei Z, Pan Z, Chen Y, Li X, Mao T, He Q, Fan D: Genetic association between p53 codon 72 polymorphism and risk of cutaneous squamous cell carcinoma. Tumour Biol 2014;35:3899-3903.

Table 1. Minor allele frequency (MAF) of the analysed SNPs in both RTR groups, our control group as well as the NCBI database. SNPs without different alleles in the analysed group are declared as n.d. and such without examination (control group only) as n.a.. Type of used method is declared in the last column.

SNP	Gene	Protein	MAF RTR group 1	MAF RTR group 3	MAF controls	MAF NCBI	type of used method
TMC6/EVER1							
rs2748427	373T>C	Trp125Arg	C=0.250/8	C=0.167/8	C=0.175/36	C=0.285/621	assay
rs12449858	457C>T	Leu153Phe	T=0.125/3	T=0.143/4	T=0.129/27	T=0.140/305	assay
rs34712518	572G>A	Gly191Asp	A=0.042/1	n.d.	A=0.042/9	A=0.096/209	assay
rs2613522	1082+5t>c	-	C=0.375/12	C=0.300/15	C=0.210/45	C=0.417/908	assay
rs1474865	1083-57c>g	-	G=0.091/2	G=0.154/4	n.a.	G=0.130/284	RFLP (StyI)
rs2057188	1083-4c>g	-	G=0.156/5	G=0.100/5	G=0.075/16	G=0.083/180	assay
rs2748428	1811+25a>g	-	A=0.437/14	A=0.360/18	G=0.465/94	A=0.275/599	assay
rs2252496	1812-54t>a	-	A=0.318/7	A=0.475/19	n.a.	A=0.412/897	RFLP (MboI)
rs79153946	2355-4g>a	-	A=0.045/1	A=0.036/1	n.a.	A=0.008/18	RFLP (Hpy1881)
rs2253277	*156G>A	3‘ UTR	A=0.125/4	A=0.180/9	A=0.083/18	A=0.066/144	assay
TMC8/EVER2							
rs383603	-239g>c	5‘ UTR	G=0.188/6	G=0.180/9	G=0.303/66	G=0.319/695	assay
rs452483	-187c>t	5‘ UTR	T=0.156/5	T=0.100/5	T=0.060/13	T=0.082/178	assay
rs417780	668+13t>c	-	C=0.182/4	C=0.179/5	n.a.	C=0.276/601	RFLP (AvaII)
rs7208422	917A>T	Asn306Ile	T=0.438/14	A=0.420/21	A=0.486/101	A=0.458/997	assay
rs62079073	988-4g>t	-	T=0.100/3	T=0.100/5	T=0.084/17	T=0.091/198	RFLP (FauI)
rs12452890	1107G>A	Glu369Glu	G=0.406/13	G=0.380/19	G=0.433/91	G=0.438/953	assay
rs112802399	1024G>T	Gly342Trp	T=0.045/1	n.d.	n.a.	T=0.011/23	RFLP (ScrFI)
rs12449680	1252-52a>g	-	G=0.344/11	G=0.220/11	G=0.252/52	G=0.373/812	assay
rs16970849	1349+13g>a	-	A=0.100/2	A=0.107/3	A=0.014/3	A=0.160/348	RFLP (Tsp45I)
rs11651675	1501G>A	Val501Ile	A=0.083/2	A=0.071/2	A=0.048/10	A=0.019/42	assay
rs11651650	1533+64c>t	-	T=0.042/1	n.d.	T=0.047/10	T=0.017/36	assay
rs11651741	1534-23g>a	-	A=0.042/1	n.d.	A=0.057/12	A=0.019/42	assay
rs11651864	1665-5g>t	-	T=0.042/1	n.d.	T=0.034/7	T=0.019/41	assay
unknown SNP	1766G>A	Arg586His	A=0.045/1	n.d.	n.a.	unknown	RFLP (SphI)
rs7221365	1826+15c>a	-	C=0.400/12	C=0.375/18	C=0.434/98	C=0.403/877	RFLP (HpaII)
rs369764	*5t>g	3‘ UTR	n.d.	n.d.	n.d.	T=0.002/4	assay
TP53							
rs1042522	c.215C>G	Pro72Arg	C=0.219/7	C=0.220/11	C=0.258/51	C=0.295/551 (1000genome)	RFLP (FauI)

Table 2. Frequency of *TMC6/EVER1*, *TMC8/EVER2* and *p53* SNPs analysed in two RTR collectives (group 1 and group 3) as well as in the control group. Named are numbers of SNPs and used type of test. In case of three different genotypes p-value was calculated by Cochran-Armitage test (CATT) and logistic regression (lrpval). In case of only two different genotypes the Fisher test (FT) was used.

SNP	Group 1 (%)	Group 3 (%)	controls	test	pval	lrpval	OR (95% CI)
TMC6							
rs2748427				CATT	0.46	0.46	
TT	11 (68.75)	18 (75.0)	70 (68.0)				
TC	2 (12.5)	4 (16.7)	30 (29.1)				0.89 (0.16 - 4.95)
CC	3 (18.75)	2 (8.3)	3 (2.9)				2.25 (0.38 - 13.34)
rs12449858				FT	1	NA	
CC	9 (75.0)	10 (71.4)	79 (75.2)				
CT	3 (25.0)	4 (28.6)	25 (23.8)				0.86 (0.17 - 4.47)
TT	0 (0)	0 (0)	1 (0.96)				NA
rs34712518				FT	0.46	NA	
GG	11 (91.7)	14 (100)	97 (91.5)				
GA	1 (8.3)	0 (0)	9 (8.5)				3.78 (0.14 - 101.83)
AA	0 (0)	0 (0)	0 (0)				NA
rs2613522				CATT	0.55	0.55	
TT	8 (50.0)	14 (56.0)	65 (60.7)				
TC	4 (25.0)	7 (28.0)	39 (36.5)				1.02 (0.24 - 4.33)
CC	4 (25.0)	4 (16.0)	3 (2.8)				1.71 (0.36 - 8.09)
rs1474865				CATT	0.55	0.55	
CC	9 (81.8)	10 (76.9)	n.a.				
CG	2 (18.2)	2 (15.4)	n.a.				1.11 (0.16 - 7.85)
GG	0 (0)	1 (7.7)	n.a.				0.37 (0.01 - 10.18)
rs2057188				CATT	0.47	0.47	
CC	11 (68.75)	21 (84.0)	90 (84.9)				
CG	5 (31.25)	3 (12.0)	16 (15.1)				2.94 (0.64 - 13.43)
GG	0 (0)	1 (4.0)	0 (0)				0.62 (0.02 - 16.56)
rs2748428				CATT	0.45	0.45	
AA	3 (18.75)	2 (8.0)	27 (26.7)				
AG	8 (50.0)	14 (56.0)	54 (53.5)				0.42 (0.07 - 2.61)
GG	5 (31.25)	9 (36.0)	20 (19.8)				0.41 (0.06 - 2.86)
rs2252496				CATT	0.98	0.98	
TT	5 (45.45)	6 (42.9)	n.a.				
TA	5 (45.45)	7 (50.0)	n.a.				0.87 (0.18 - 4.21)
AA	1 (9.1)	1 (7.1)	n.a.				1.18 (0.09 - 14.87)
rs79153946				FT	1	NA	
GG	10 (90.9)	13 (92.9)	n.a.				
GA	1 (9.1)	1 (7.1)	n.a.				1.29 (0.12 - 14.21)
AA	0 (0)	0	n.a.				NA
rs2253277				CATT	0.54	0.53	
GG	12 (75.0)	18 (72.0)	90 (83.3)				
GA	4 (25.0)	5 (20.0)	18 (16.7)				1.21 (0.29 - 5.10)
AA	0 (0)	2 (8.0)	0 (0)				0.30 (0.01 - 6.70)
TMC8/EVER2							
rs383603				CATT	0.93	0.93	
GG	0 (0)	1 (4.0)	11 (10.1)				
GC	6 (37.5)	7 (28.0)	44 (40.4)				2.6 (0.09 - 75.50)
CC	10 (62.5)	17 (68.0)	54 (49.5)				1.8 (0.07 - 48.36)
rs452483				CATT	0.47	0.47	
CC	11 (68.75)	21 (84.0)	96 (88.1)				
CT	5 (31.25)	3 (12.0)	13 (11.9)				2.94 (0.65 - 13.43)
TT	0 (0)	1 (4.0)	0 (0)				0.62 (0.02 - 16.56)
rs417780				CATT	0.98	0.98	
tt	7 (63.6)	10 (71.4)	n.a.				

tc	4 (36.4)	3 (21.4)	n.a.				1.8 (0.34 - 9.68)
cc	0 (0)	1 (7.1)	n.a.				0.47 (0.02 - 13.10)
rs7208422				CATT	0.15	0.15	
AA	3 (18.75)	4 (16.0)	27 (26.0)				
AT	12 (75.0)	13 (52.0)	47 (45.2)				1.19 (0.25 - 5.87)
TT	1 (6.25)	8 (32.0)	30 (28.8)				0.23 (0.02 - 2.11)
rs62079073				CATT	1	1	
GG	12 (80.0)	22 (88.0)	84 (83.2)				
GT	3 (20.0)	1 (4.0)	17 (16.8)				4.2 (0.55 - 32.10)
TT	0 (0)	2 (8.0)	0 (0)				0.36 (0.02 - 8.11)
rs12452890				CATT	0.81	0.81	
GG	2 (12.5)	4 (16.0)	18 (17.1)				
GA	9 (56.25)	11 (44.0)	55 (52.4)				1.49 (0.25 - 8.72)
AA	5 (31.25)	10 (40.0)	32 (30.5)				0.94 (0.15 - 6.05)
rs112802399				FT	0.44	NA	
GG	10 (90.9)	14 (100)	n.a.				
GT	1 (9.1)	0 (0)	n.a.				4.14 (0.15 – 112.07)
TT	0 (0)	0 (0)	n.a.				NA
rs12449680				CATT	0.19	0.19	
AA	6 (37.5)	15 (60.0)	58 (56.3)				
AG	9 (56.25)	9 (36.0)	38 (36.9)				2.38 (0.66-8.61)
GG	1 (6.25)	1 (4.0)	7 (6.8)				2.38 (0.21 - 27.40)
rs16970849				FT	1	NA	
GG	8 (80.0)	11 (78.6)	105 (97.2)				
GA	2 (20.0)	3 (21.4)	3 (2.8)				0.97 (0.15 - 6.14)
AA	0 (0)	0 (0)	0 (0)				NA
rs11651675				FT	1	NA	
GG	10 (83.3)	12 (85.7)	94 (90.4)				
GA	2 (16.7)	2 (14.3)	10 (9.6)				1.19 (0.17 - 8.25)
AA	0 (0)	0 (0)	0 (0)				NA
rs11651650				FT	0.46	NA	
CC	11 (91.7)	14 (100)	96 (90.6)				
CT	1 (8.3)	0 (0)	10 (9.4)				3.78 (0.14 - 101.83)
TT	0 (0)	0 (0)	0 (0)				NA
rs11651741				FT	0.46	NA	
GG	11 (91.7)	14 (100)	95 (89.7)				
GA	1 (8.3)	0 (0)	10 (9.4)				3.78 (0.14 - 101.83)
AA	0 (0)	0 (0)	1 (0.9)				NA
rs11651864				FT	0.46	NA	
GG	11 (91.7)	14 (100)	96 (93.2)				
GT	1 (8.3)	0 (0)	7 (6.8)				3.78 (0.14 - 101.83)
TT	0 (0)	0 (0)	0 (0)				NA
unknown SNP				FT	0.44	NA	
GG	10 (90.9)	14 (100)	n.a.				
GA	1 (9.1)	0 (0)	n.a.				4.14 (0.15 - 112.07)
AA	0 (0)	0 (0)	n.a.				NA
rs7221365				CATT	0.82	0.82	
CC	2 (13.3)	3 (12.5)	23 (20.4)				
CA	8 (53.3)	12 (50.0)	52 (46.0)				0.95 (0.15 - 6.01)
AA	5 (33.3)	9 (37.5)	38 (33.6)				0.81 (0.12 - 5.60)
rs369764				NA	NA	NA	
TT	0 (0)	0 (0)	0 (0)				
GT	0 (0)	0 (0)	0 (0)				NA
GG	12 (100)	13 (100)	107 (100)				NA
TP53							
rs1042522				CATT	0.99	0.99	
CC	2 (12.5)	1 (4.0)	5 (5.1)				
CG	3 (18.8)	9 (36.0)	41 (41.4)				0.22 (0.02 – 2.36)
GG	11 (68.8)	15 (60.0)	53 (53.5)				0.45 (0.05 – 3.87)