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RESEARCH ARTICLE

Transcriptional activity of the *RHOB* gene is influenced by regulatory polymorphisms in its promoter region

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Abstract Osteoarthritis (OA) is a chronic joint disease with genetic as well as environmental factors contributing to its etiology. We recently identified *RHOB* as a gene overexpressed in osteoarthritis. Interestingly, *RHOB* harbors numerous polymorphisms in its promoter region and genotyping of OA patients and healthy controls revealed an association of the single nucleotide polymorphism (SNP) rs585017 with the disease. We here set out to investigate the influence of *RHOB* promoter polymorphisms on the transcriptional activity of the gene and we found evidence that the SNPs rs2602160 and rs585017 cooperate in regulating *RHOB* expression. In addition, a variable number of tandem repeats (VNTR) impacts on the *RHOB* transcriptional activity in a cell type restricted manner. These results mechanistically link our previous finding of an elevated *RHOB* expression to the disease associated SNP rs585017 and confirm a role for regulatory polymorphisms in osteoarthritis.

Keywords Chondrocytes · Gene expression · Osteoarthritis · Regulatory polymorphisms

Abbreviations

OA	Osteoarthritis
SNP	Single nucleotide polymorphism
TR	Tandem repeats
PCR	Polymerase chain reaction

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Introduction

Osteoarthritis (OA, [MIM 165720]) is the most prevalent joint disease in the elderly and considered a complex disease including genetic as well as environmental components (Buckwalter et al. 2004; Hunter et al. 2002). It is characterized by the complete loss of articular cartilage, leaving the affected joints functionally impaired. In the last decade, several genetic susceptibility factors were identified, among them regulatory gene polymorphisms in the *IL-1* locus (Moos et al. 2000; Spector and MacGregor 2004). We recently described the gene *RHOB* (MIM 165370) as a novel susceptibility gene for OA (Mahr et al. 2006). *RHOB* was overexpressed in OA patients compared to healthy controls and genotyping of larger cohorts revealed a disease association of the G allele of the SNP rs585017 located at position −165 in the *RHOB* promoter. *RHOB* encodes a small GTPase involved in a broad range of cellular activities, e.g. actin organization, vesicular transport, stress response, and apoptosis (Prendergast 2001; Ridley 2001). Sequence analysis of the *RHOB* promoter region revealed the presence of additional polymorphisms including several SNPs (unpublished data) and a VNTR sequence (Tovar et al. 2003). We here used Luciferase reporter assays to investigate *RHOB* promoter polymorphisms for their functional impact on transcriptional regulation.

Materials and methods

Subjects

Genotyping of the VNTR polymorphism was performed on 147 OA patients undergoing joint replacement surgery and

179 healthy controls. Details about study subjects are published elsewhere (Mahr et al. 2006). The study was approved by the local ethical committee and appropriate informed consent was obtained from all human subjects.

Cell culture

Both, the chondrosarcoma line SW1353 and HeLa cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (Invitrogen). For transient transfections, cells were grown in 6-well-plates at a concentration of 1×10^6 cells per well using 2 ml DMEM supplemented with 10% FCS without antibiotics.

Genotyping of the VNTR polymorphism

The VNTR polymorphism was genotyped via genomic polymerase chain reaction (PCR) using the primers 5′-tcggttgacttgggtctttc-3′ (forward) and 5′-cactatgaactctgatcccg-3′ (reverse) followed by electrophoretical separation on a 3% NuSieve® 3:1 agarose gel (Biozym).

DNA sequencing

Sequencing analysis was done as described previously (Mahr et al. 2006).

Analysis of transcriptional activity

Two 5′-flanking regions of *RHOB* (−1469 to −1223 and −1224 to + 109) harboring the respective SNPs rs2602160 and rs585017 as well as the VNTR element were prepared by PCR. Genomic DNA containing the CC or TT genotype at −1285 bp and the genotype AA or GG at −165 bp from the transcription start site was used as a PCR template. For amplification of the most 5′ region, the primers 5′-nnnnn-acgcgtcaccttcctgcttgtgttca−3′ (forward) containing a *MluI* restriction site and 5′-tagaatccaaccaccccaag-3′ (reverse) were used. The region from −1224 to +109 was amplified using the primers 5′-tagaggggacacccctaac-3′ (forward) and 5′-nnnnnagatct-ctcccgggtctctccg-3′ (reverse) containing a *BglII* restriction site. The PCR products were inserted into the luciferase reporter vector pGL3-enhancer (Promega). We combined the different genotypes of the SNPs rs2602160 and rs585017 using a *NheI* restriction site. SW1353 cells and HeLa cells were transiently transfected using 4 µl FuGENE HD and 400 ng of the pGL3-construct. Cells were cotransfected with 25 ng of a constitutively active *Renilla* luciferase vector (pRL-TK) and firefly luciferase activity was normalized to *Renilla* luciferase activity. A total of 24 h after transfection, cells were lysed using 500 µl Passive Lysis Buffer (Promega). Luciferase activities from firefly or *Renilla* were measured using a Dual-Glo Luciferase Assay System (Promega) and the luminometer Lumat LB 9501 (Bertold).

Statistical analysis

Student's t-test including Welch's correction was used to determine the significance of differences in the luciferase reporter assays.

Results

Cooperative regulation of promoter SNPs

Sequence analyses of the *RHOB* promoter regions of six OA patients and six healthy controls confirmed the presence of SNPs at positions −165 (rs585017) and at −1285 (rs2602160) (Fig. 1a). Interestingly, both SNPs showed an identical distribution suggesting the possibility of linkage disequilibrium. To test whether the OA associated SNP at −165 itself or the one at −1285 influence the transcriptional activity of *RHOB*, four different luciferase constructs were established carrying either the wildtype-haplotype (pGL3-TA), mixed haplotypes (pGL3-TG and pGL3-CA), or the SNP-haplotype (pGL3-CG). All four constructs were analyzed in the chondrocyte-like cell line SW1353 and in HeLa cells and the results show that the SNP-haplotype (pGL3-CG) yielded a significantly higher transcriptional activity compared to the wildtype-haplotype (pGL3−TA) with *P*-values of 0.008 and 0.02 in SW1353 and HeLa cells, respectively (Fig. 1b, c). Interestingly, neither the rs2602160 C allele nor the rs585017 G allele by themselves were able to influence the transcriptional activity of *RHOB* (data not shown). Out data therefore suggest that the rs2602160 and rs585017 promoter SNPs cooperate in regulating the transcriptional activity of *RHOB*.

Cell type-restricted regulation of the VNTR polymorphism

The human *RHOB* promoter also harbors a VNTR polymorphism which is located 820 bp upstream of the transcriptional start site and which carries between 8 and 14 numbers of a 34 bp tandem repeat (Fig. 1a) (Tovar et al. 2003). To test if OA patients and healthy controls differ in their VNTRs, we analyzed larger cohorts via PCR

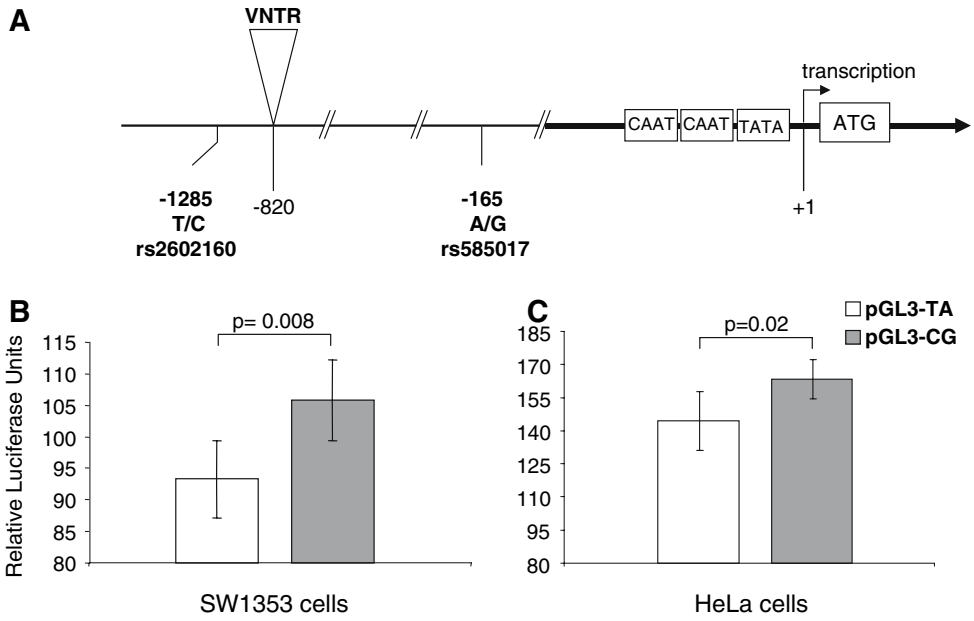

Fig. 1 Cooperative regulation of the *RHOB* promoter SNPs rs585017 and rs2602160. (**a**) Location of the *RHOB* promoter polymorphisms as determined by DNA sequence analysis. CAAT- and TATA-boxes are indicated as is the transcriptional start site. (**b, c**) Influence of the promoter SNPs on the transcriptional activity of the *RHOB* in SW1353 cells (**b**) and in HeLa cells (**c**). The vectors pGL3-TA and pGL3-CG carry the wildtype- (T at the position −1285 and A at the position −165 bp) and the SNP-haplotyp (C at position −1285 and G at position −165), respectively. Each experiment was performed six times; relative luciferase units are given as mean ± SD

amplification. For both healthy controls and OA patients the most frequently carried number of TR was 10 and there was no statistically significant difference in the distribution between both groups (Table 1). However, we did find a statistically significant increase in G alleles of rs585017 in patients carrying 10 TR compared to carriers of any other number of TR (data not shown). To test, if the VNTR influences the transcriptional activity under the control of the *RHOB* wildtype-haplotype promoter, we established 2 different reporter constructs harboring either a short VNTR element with 9 (pGL3-9TR) or a long element with 13 repeats (pGL3-13TR). Interestingly, the long promoter construct pGL3-13TR revealed a significantly higher transcriptional activity in SW1353 cells compared with the short construct pGL3-9TR. The *P*-value of 0.007 indicates statistical significance (Fig. 2a) however, these results could not be confirmed in HeLa cells suggesting a cell type restricted regulatory mechanism (Fig. 2b).

Table 1 Distribution of the numbers of tandem repeats in the VNTR sequence of the human *RHOB* promoter

Number of repeats	Control alleles (n = 358)	Patient alleles (n = 294)
8	0	1
9	28	16
10	173	163
11	116	72
12	8	10
13	29	29
14	4	3

Discussion

In the present study we demonstrate that the promoter SNPs rs585017 and rs2602160 cooperate in increasing the transcriptional activity of *RHOB* in both, a chondrosarcoma line and in HeLa cells. The VNTR sequence located 820 bp upstream of the transcriptional start site also influences *RHOB* expression in that an elevated number of TR increases the transcriptional activity. However, this latter regulation is restricted to the chondrosarcoma line.

The synergistic effect of the promoter SNPs is likely to be provoked by conformational changes in the secondary structure of the DNA which in turn may modify the binding of transcription factors. Indeed, the presence of the rs2602160 SNP-haplotype generates a consensus recognition site for the nuclear factor 1 (NF-1) which is absent in the presence of the wildtype-haplotype. We did not find obvious transcription factor binding sites at rs585017 however, there may be cooperative binding of transcription factors which is also dependent on a conformational change at −165.

Genotype analyses revealed no association of the VNTR polymorphism with OA. However, we here show that a VNTR sequence carrying 13 repeats increases the transcriptional activity of *RHOB* compared to a VNTR with only 9 repeats. Our findings may seem contradictory to what has been published before (Tovar et al. 2003): Tovar et al. analyzed the effect of the VNTR sequence in combination with the SV40 promoter and did not find an impact of the number of repeats on the transcriptional activity. We here investigated the VNTR in combination with its native *RHOB* promoter and we used the chondrocyte-like

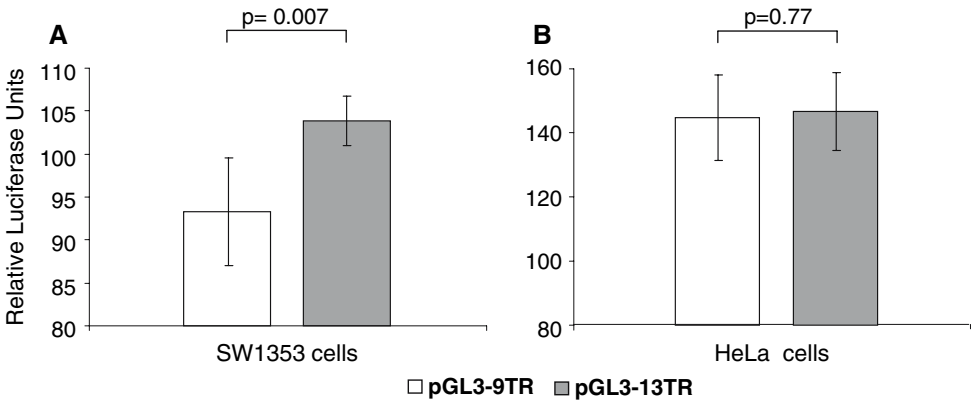

Fig. 2 Functional analysis of the VNTR sequence within the *RHOB* promoter. (**a**) Influence of different TR numbers on the transcriptional activity of the *RHOB* gene in SW1353 cells (**a**) and in HeLa cells (**b**). *RHOB* wildtype-haplotype promoter sequences including 9 (pGL3-9TR) or 13 TRs (pGL3-13TR) were cloned into the pGL3-enhancer vector. Each experiment was performed six times; relative luciferase units are given as mean ± SD

SW1353 and HeLa cells while it was human MCF7 cells in the Tovar study. As both HeLa and MCF7 cells are of epithelial origin, a cell type restricted regulation of the VNTR polymorphism on *RHOB* gene expression is likely.

RHOB encodes a small GTPase which is involved in a broad range of cellular processes like vesicular transport, actin organization and apoptosis (Prendergast 2001; Ridley 2001) and indeed, we recently described a significant increase of apoptosis in *RHOB* overexpressing chondrocyte-like SW1353 cells (Mahr et al., submitted manuscript). Our data in combination with published reports describing significant apoptosis of the articular chondrocytes during OA (Blanco et al. 1998; Thomas et al. 2007) point at a functional role of *RHOB* overexpression in OA pathogenesis. Because OA is a disease of the elderly, we speculate that even a slightly enhanced *RHOB* expression over decades would influence the physiology of the articular chondrocytes leading to cell death and a loss of articular cartilage.

Taken together our data indicate that both the SNPs and the VNTR within the promoter region of the *RHOB* gene are true regulatory polymorphisms which via differential expression contribute to OA pathogenesis.

Conclusion

Here, we describe the synergistic effect of two single nucleotide polymorphisms in increasing the transcriptional activity of the gene RHOB previously described to be associated with osteoarthritis. Additionally, we found that a VNTR sequence also influences RHOB expression in that an elevated number of tandem repeats increases the transcriptional activity. Together, our data indicate that both the SNPs and the VNTR are true regulatory polymorphisms which via differential expression of the RHOB gene contribute to osteoarthritis pathogenesis.

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