# An intronic COL1A1 Sp1-Polymorphism but not Vitamin D Receptor Gene Polymorphisms Relates to the Phenotypic Expression of Osteogenesis imperfecta

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[Electronic ed. 2022, Münster: Westfälische Wilhelms–Universität] DOI: 10.17879/05099611989

## Abstract

This is the first observation of a polymorphism associated with the severity of clinical manifestation in Osteogenesis imperfecta (OI). The results are consistent with a polygenic quantitative trait model in which OI albeit a monogenic autosomal dominant disease can be modified by additional risk factors. In our collection the severe cases of OI are significantly associated with a COL1A1 Sp1 polymorphism, an association initially observed in postmenopausal women with osteoporosis. In both studies a G → T polymorphism in the same Sp1 motif is found rather in the clinically severe cases. As diverse vitamin D receptor (VDR)-polymorphisms are one of the strongest risk factors of osteoporosis this prompted us to examine a putative association in our OI collection. However, we could not find a correlation of the severity of OI with any of the four polymorphisms (*Bsm*I, *Apa*I, and *Taq*I RFLPs from intron 8 to exon 9, and *Fok*I RFLP in exon 2). The number of OI subjects may be insufficient to address the effect of VDR adequately. We conclude that the COL1A1 Sp1 polymorphism is of high prognostic value in OI individuals homozygous for the T allele (odds ratio 10.18, 95% confidence interval 1.021 to 101.5, p = 0.0479).

**Key words** Osteogenesis imperfecta, COL1A1, COL1A2, Sp1 polymorphism, VDR gene polymorphisms

## Introduction

Collagen type I is the major protein in bone. Hence, it is not surprising that Osteogenesis imperfecta (OI) is mostly related to mutations in one of the coding genes COL1A1 and COL1A2 [1]. There are no mutational hot spots allowing a clear genotype/phenotype correlation. Only 20% of the patients share common mutations [2]. An increasing number of exceptions of a „gradient model" [3] which relates severe forms of OI to mutations rather C-terminal and mild ones rather N-terminal of the helical domain also do not support in any case a „regional model" [4,44]. This indicates the presence of environmental and/or modifying genetic factors. A preliminary support for this assumption is given by the observations of intrafamilial variability or of non-related patients with an identical mutation but different phenotypes [5,6].

A COL1A1 Sp1 polymorphism was found to be associated with osteoporosis in postmenopausal women [7]. To emphasize the contribution of this factor to other bone diseases especially to those related to the same gene, we studied patients with different types of OI. The prevalence of the individual alleles should be the same in controls as in patients with OI. However, those polymorphic alleles which are thought to be significantly associated with reduced bone density and increased fracture rate should be rather found among the severe types of OI. Attention is also being directed to VDR gene polymorphisms whose association to osteoporosis was shown [8-16] but not confirmed in all studies [17-19].

## Patients and Methods

Subjects

The study comprised 72 unrelated individuals with OI and a control group of 100 unrelated probands all of Caucasian origin, respectively. A clinical follow-up of up to 10 years [20-22] allowed an optimal classification according to Sillence et al. [23]. Those who did not fit the criteria unambiguously (12.5%, n = 9) were categorized as „unclassified" (Table 1). The clinical manifestation of OI in this latter group was „moderate" and thus they were summarized with OI types III/IV and IV.

**Table 1** Distribution of patients and controls

| **Classification of Sillence** | **N** | **simplified clinical ranking** | **N** |
|---|---|---|---|
| **OI I** | 18 | mild | 18 |
| **OI II** | 10 | | |
| **OI II/III** | 2 | | |
| **OI III** | 13 | severe | 25 |
| **OI III/IV** | 3 | | |
| **OI IV** | 17 | | |
| **OI, unclassified** | 9 | moderate | 29 |
| **Total** | 72 | | 72 |
| **Controls** | 100 | | |

Genotyping

DNA was prepared from cultured fibroblasts, osteoblasts, or whole blood samples according to standard procedures. 100 ng of genomic DNA was amplified in a 50 µl volume. Oligonucleotide primers were designed from published sequence data flanking the polymorphic sites of COL1A1 Sp1 [7] and of VDR exons 2 [12], and 7 to 9 [9,16]. In case of COL1A1 Sp1 a *Bal*I site was introduced for the T-polymorphism by a mismatch primer.

*Sp1 polymorphism:* The PCR protocols given by Grant et al. [7] were adapted for a GeneAmp PCR System 9600® (Perkin Elmer, Weiterstadt, Germany): Denaturation 4 minutes 95°C followed by 40 cycles 50 s 94°C, 10 s 64° followed by ramping at 1°C per 10 s to 72°C, 15 s 72°C. A final elongation step at 72°C for 300 s was added. The PCR products were digested with *Bal*I (from Promega, Madison, WI, USA, all other enzymes from New England Biolabs, Beverly, MA, USA) at least for for 2 h at 37°C.

*VDR gene polymorphisms:* The DNA amplifications were done on a RoboCycler® (Stratagene, Heidelberg, Germany) as following: *Bsm*I alleles B and b (capital letter documents absence of restriction site) 95°C 30 s, 72°C 90 s, the *Apa*I, *Taq*I alleles (A, a and T, t) 95°C 30 s, 65°C 30 s, 72°C 120 s, and *Fok*I alleles (F, f) 95°C 30 s, 60°C 30 s, 72°C 30 s followed by 35 (alleles B, b, A, a, T, t) or 30 (F,f) cycles with a preceding denaturation of 95°C 240 s and a final elongation step at 72°C 300 s (B, b, F, f) and 420 s (A, a, T, t), respectively. The digests were done for a minimum of 3 h. All products were analyzed on a 2% agarose gel after staining with ethidium bromide (Figures 1, 2)

**Figure 1** Overview of the polymorphic VDR gene sites used in this study. The *Fok*I RFLP creates a potential second translation start codon by a C → T transition indicated by the underlined partial sequence.

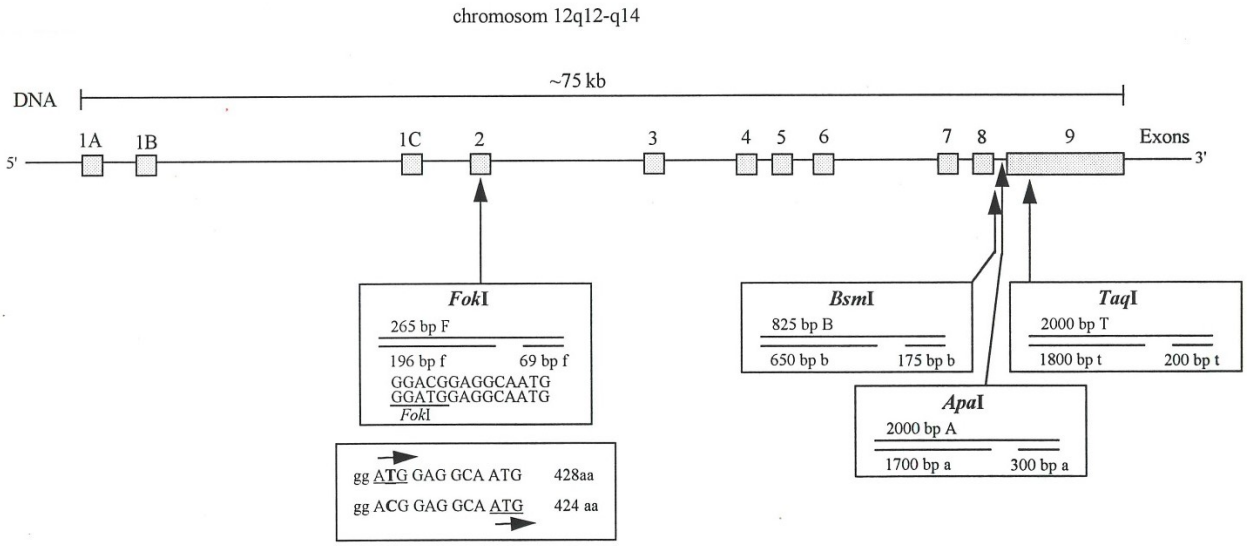
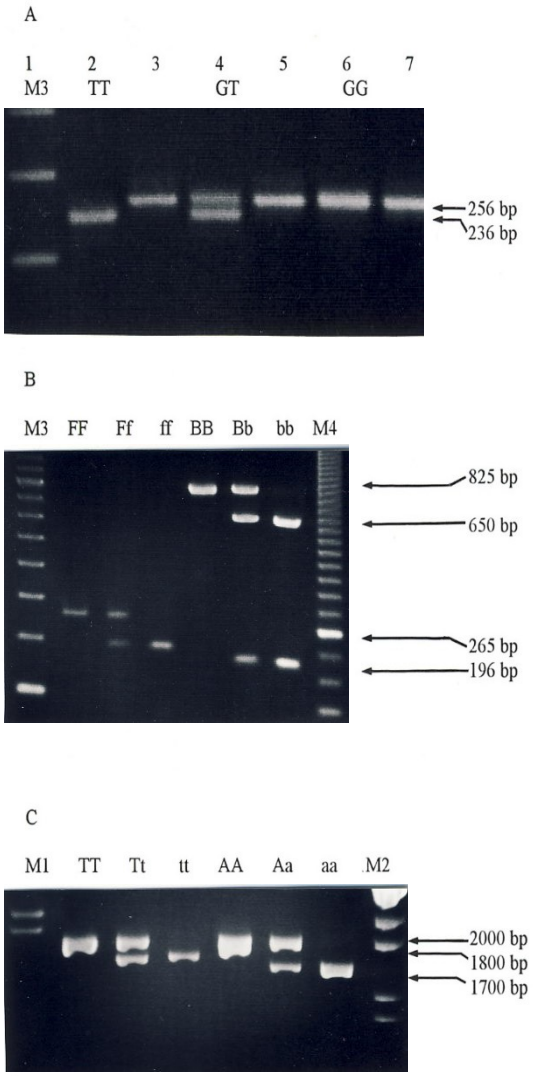


**Figure 2** Examples of genotyping reactions for A) COL1A1 Sp1 polymorphism, lane 3, 5, and 7 are showing non digested controls, B) VDR polymorphisms *Fok*I and *Bsm*I RFLPs, C) VDR polymorphisms (*Apa*I, *Taq*I). The molecular weight markers are M 1 (Lambda *Bst*EII), M2 (Lambda *Hind*III), M3 (100 bp ladder, Amersham Pharmacia Biotech, Freiburg, Germany), M4 (50 bp ladder, Amersham Pharmacia Biotech, Freiburg, Germany). Genotypes are as indicated at the top of each lane. The fragment lenghts [bp] of only the relevant RFLPs are given on the right side.



Statistical analysis

Tests for Hardy-Weinberg equilibrium were performed by comparing observed and expected genotypes. Linkage disequilibrium between the different polymorphic sites and the different ethnic groups was assessed by chi-square test. Genotypes were entered as a categorical factor in a logistic regression analysis to evaluate ist impact on the severity of disease. Analyses were conducted using StatView® (SAS Institute Inc., USA, 1998).

## Results

*COL1A1 Sp1 polymorphism*

The prevalence of the genotypes in the OI collection (54.2 % GG, 38.8% GT, 7.0% TT, n = 72) was the same as in the control group (65 % GG, 30 % GT, 5% TT, n = 100). The observed and expected frequencies in the OI group ($\chi^2$ = 0.0, p = 1.000), in the control group ($\chi^2$ = 0.18, p = 0.912), and also the observed frequencies between both groups ($\chi^2$ = 2.07, p = 0.356) were in full agreement with the Hardy-Weinberg equilibrium suggesting no collection bias (Table 2).

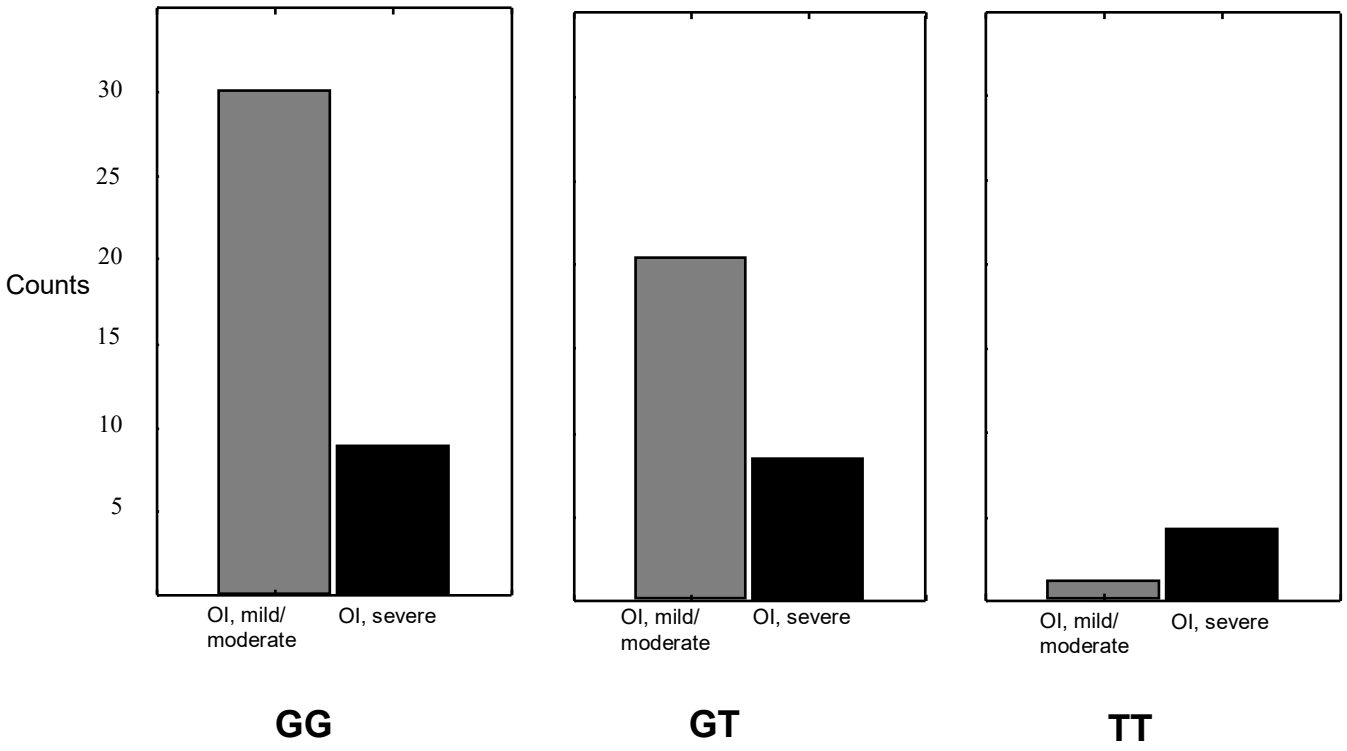
**Table 2** Haplotype frequencies of the COL1A1 Sp1 polymorphism

| Polymorphisms | OI | | | Controls | | |
|---|---|---|---|---|---|---|
| ***COL1A1 Sp1*** | | | | | | |
| | (n = 72) | | | (n = 100) | | |
| | observed | expected* | p-test for H.-W.E | observed | expected* | p-test for H.-W.E. |
| GG | .542 | .542 | | .650 | .640 | |
| GT | .388 | .388 | $\chi^2$ = 0.00 | .300 | .320 | $\chi^2$ = 0.18 |
| TT | .070 | .070 | p = 1.000 | .050 | .040 | p = 0.912 |
| ***VDR*** | (n = 72) | | | (n = 54) | | |
| *Bsm*I RFLP | | | | | | |
| BB | .222 | .180 | | .260 | .250 | |
| Bb | .403 | .488 | $\chi^2$ = 1.05 | .480 | .500 | $\chi^2$ = 0.04 |
| bb | .375 | .332 | p = 0.592 | .260 | .250 | p = 0.982 |
| *Apa*I RFLP | | | | | | |
| AA | .222 | .257 | | .280 | .320 | |
| Aa | .570 | .500 | $\chi^2$ = 0.70 | .570 | .490 | $\chi^2$ = 0.69 |
| aa | .208 | .243 | p = 0.705 | .150 | .190 | p = 0.710 |
| *Taq*I RFLP | | | | | | |
| TT | .375 | .372 | | .296 | .348 | |
| Tt | .472 | .476 | $\chi^2$ = 0.00 | .593 | .484 | $\chi^2$ = 1.45 |
| tt | .153 | .152 | p = 0.999 | .111 | .168 | p = 0.480 |
| *Fok*I RFLP | | | | | | |
| FF | .389 | .390 | | .296 | .330 | |
| Ff | .472 | .469 | $\chi^2$ = 0.00 | .556 | .489 | $\chi^2$ = 0.51 |
| ff | .139 | .141 | p =1.000 | .148 | .181 | p = 0.775 |

* Expected under the assumption of Hardy-Weinberg equilibrium (H.-W. E.)

When estimating separately for the most severe types of OI (i.e. types II and III) vs. the combined mild and moderate forms of OI there was a strong relation to the COL1A1 Sp1 polymorphism with preponderance of the T alleles. The risk for developing severe type of OI is for the TT genotype greater than for the GG genotype (Table 3) (odds ratio 10.18, 95% confidence interval 1.021 to 101.5, p = 0.0479).

**Table 3** Frequency correlation of the COL1A1 SP1 alleles G and T, and the two classes of mild/moderate and severe Osteogenesis imperfecta (OI).




The GT genotype showed also a tendency to the severe type of OI but was not statistically significant (odds ratio 1.414, 95% confidence interval 0.499 to 4.006, p = 0.5142). For more subtle intergroup differences based on the Sillence classification {#34} or a simplified scheme „mild", „moderate", „severe" OI (Table 1) statistical power was not strong enough to detect significance in a study group of this size. For comparison, the haplotype frequencies of three distinct populations published by Grant et al. [7] and Uitterlinden et al. [24] were also in Hardy-Weinberg equilibrium (Table 4a) but only the Aberdeen group showed the same distribution as our control group (Table 4b).

**Table 4**

**a)** Summary of all published haplotype frequencies of the COL1A1 Sp1 polymorphism. They are all in Hardy-Weinberg equilibrium.

| Polymorphisms | Population-based studies | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| ***COL1A1 Sp1*** | British, Aberdeen pre-/postmenopausal females normal/osteoporosis Grant et al. [7] | | | British, London postmenopausal females normal/osteoporosis Grant et al. [7] | | | Dutch, Rotterdam, postmenopausal females, normal/osteoporosis Uittterlinden et al. [34] | | |
| | n = 205 | | | n = 94 | | | n = 1778 | | |
| | observed | expected* | p-test for H.-W. E. | observed | expected* | p-test for H.-W. E. | observed | expected* | p-test for H.-W. E. |
| GG | .605 | .609 | | .670 | .689 | | .671 | .673 | |
| GT | .351 | .343 | $\chi^2 = 0.06$ | .319 | .282 | $\chi^2 = 1.04$ | .296 | .295 | $\chi^2 = 0.02$ |
| TT | .044 | .048 | p = 0.971 | .011 | .029 | p = 0.594 | .033 | .032 | p = 0.992 |

* Expected under the assumption of Hardy-Weinberg equilibrium (H.-W. E.)

**b)** P-test for frequencies observed in our collection and in the published populations, respectively. The allele frequencies from the London and Rotterdam groups differ from our study group.

| | This study | Aberdeen | London |
|---|---|---|---|
| Aberdeen | $\chi^2 = 1.26$<br>p = 0.533 | - | - |
| London | $\chi^2 = 5.56$<br>p = 0.062 | $\chi^2 = 2.77$<br>p = 0.251 | - |
| Rotterdam | $\chi^2 = 6.51$<br>p =0.039 | $\chi^2 = 3.79$<br>p = 0.150 | $\chi^2 = 1.53$<br>p = 0.465 |

*VDR gene polymorphisms*

The genotypes of the OI patients and of the controls were in Hardy-Weinberg equilibrium (Table 2). Among the 27 possible VDR haplotypes 7 were observed in the OI group and 8 in the control group. Three combined haplotypes could be delineated unambiguously in the OI group as they were homozygous baT (20.8%), BAt (13.9%), and bAT (1.4%) (Table 5).

**Table 5** Frequencies and distribution of the combined VDR genotypes

| | OI study group | | Controls | |
|---|---|---|---|---|
| Genotype | N | [%] | N | [%] |
| BbAaTt | 29 | 40.3 | 23 | 42.7 |
| bbaaTT | 15 | 20.8 | 8 | 14.8 |
| bbAaTT | 11 | 15.3 | 4 | 7.4 |
| BBAAtt | 10 | 13.9 | 6 | 11.1 |
| BBAATt | 5 | 6.9 | 8 | 14.8 |
| BBAatt | 1 | 1.4 | - | - |
| bbAATT | 1 | 1.4 | 1 | 1.8 |
| bbAaTt | - | - | 1 | 1.8 |
| BbAaTT | - | - | 3 | 5.6 |
| Total | 72 | 100 | 54 | 100 |

The distribution and frequency in the control group was the same baT (14.8%), BAt (11.1%), and bAT (1.8%). An association between the type of OI and the individual or combined VDR genotypes was not apparent. There is a homologous distribution of frequencies among our control group and the combined Caucasian study groups whereas Asian and African populations showed different frequencies of the respective polymorphism (Table 6). Only in the group of Southamericans two RFLPs *Apa*I and *Fok*I were like in our control group. Comparing the combined Caucasian population with other ethnicities the *Apa*I allele in the Southamericans and the *Bsm*I allele in the Africans showed similar distribution whereas the other allele frequencies were different.

**Table 6** Comparison of VDR haplotype frequencies observed in our control group with combined non-osteoporotic populations. P-values lower than 0.050 indicate significant differences.

| Ethnicity | VDR RFLPs | $\chi^2$ test | p-value | References |
|---|---|---|---|---|
| Our control group: | | | | |
| vs. Caucasian | *Bsm*I | 3.68 | 0.158 | [8, 11, 19, 37, 43-46] |
| | *Apa*I | 1.82 | 0.403 | |
| | *Taq*I | 2.49 | 0.288 | |
| vs. Southamerican | *Bsm*I | 11.87 | 0.003 | [12, 15, 16] |
| | *Apa*I | 2.18 | 0.336 | |
| | *Taq*I | 9.21 | 0.010 | |
| | *FokI* | 0.95 | 0.622 | |
| vs. Asian | *Bsm*I | 163.17 | 0.000 | [13,46,47] |
| | *Apa*I | 30.38 | 0.000 | |
| | *Taq*I | 104.93 | 0.000 | |
| | *Fok*I | 11.36 | 0.003 | |
| vs. African | *Bsm*I | 8.55 | 0.014 | [14, 48] |
| | *Fok*I | 16.52 | 0.000 | |
| Caucasian vs. Southamerican | *Bsm*I | 14.43 | 0.001 | |
| | *Apa*I | 2.23 | 0.327 | |
| | *Taq*I | 19.17 | 0.000 | |
| Caucasian vs. Asian | *Bsm*I | 329.83 | 0.000 | |
| | *Apa*I | 148.87 | 0.000 | |
| | *Taq*I | 367.35 | 0.000 | |
| Caucasian vs. African | *Bsm*I | 4.51 | 0.105 | |

## Discussion

This is the first study demonstrating an association of a polymorphic COL1A1 Sp1 motif in intron 1 with the severity of OI. The strong genetic component of the peak bone mass is well documented [24, 25] and a variety of candidate genes were delineated which might effect the regulation of bone turnover [26, 28-32]. We concentrated on COL1A1 and COL1A2 genes coding collagen type I the most abundant protein in bone, and there is evidence that its metabolism is related to the genetic regulation of bone density [26]. 90% of patients with OI have a mutation in one of the two genes. In a prospective study Spotila et al. [27] found 3 subjects heterozygous for mutations in COL1A1 (Pro27Ala, n = 2) or COL1A2 (Ser661Gly) among 26 patients with isolated osteoporosis. In one family the consanguineous parents which were heterozygous for a 4 bp deletion in COL1A2 exhibited only osteoporosis whereas the homozygous child had OI [33]. Osteoporosis is also associated with a COL1A1 Sp1 polymorphism, which might functionally be based on an enhancer effect on the transcription of the COL1A1 gene. This was shown by Rossouw et al. [35] when constructing chimeric genes containing different segments of the human pro α1(I) collagen gene promotor. Four putative Sp1 binding sites are clustered in the central region of intron 1. In one of these Sp1 recognition motives Grant et al. [7] found a G → T polymorphism, which was significantly associated with reduced bone density in postmenopausal women. Individuals homozygous for the T alleles showed a lower bone

density on an average than normals, and heterozygotes an intermediate value. These findings were confirmed in a larger population-based study by Uitterlinden et al. [34].

The clinical overlap of osteoporosis and OI prompted us to test this COL1A1 Sp1 polymorphism in a collection with different types of OI. Genetic analyses were conducted on samples collected for purposes other than those of interest in the present study [20-22]. The observed frequencies in the whole OI study group and in the control group are very similar and are in full Hardy-Weinberg equilibrium demonstrating representative collections. However, when concentrating on the severe types of OI there is an increased prevalence of COL1A1 Sp1-T alleles as compared to the mild and modest forms. Although the major effect on OI is a mutation in the coding region of one of the COL1A1 or COL1A2 genes [1] the Sp1 polymorphism also might contribute to the OI phenotype. Whether this particular Sp1 polymorphism is just in linkage disequilibrium with a nearby disease locus or of functional relevance is still a subject of speculation. An attractive hypothesis to explain the observed association is that it alters the protein-DNA binding site. Two of the four Sp1 motives have a relative high and two a medium affinity for Sp1 [35]. But only one of them with medium affinity is harboring the polymorphism. We have sequenced the promotor region of three unrelated OI patients with very severe osteoporosis - not accounting to secondary inactivation - and have not found a base substitution (data not shown) in any of the six respective Sp1 motives.

We extended these studies to the vitamin D receptor (VDR) gene polymorphisms, which was not only related to bone mass density (BMD), osteoarthritis [36], and hyperparathyroidism [37], but also to prostate cancer [38]. We could not find an association of any of the alleles studied in our collection of OI patients. There was also no evidence for a genetically additive effect on phenotype across the four genotypes.

After the initial finding of a significant association of VDR gene polymorphisms and the serum level of circulating osteocalcin [8] this *Bsm*I RFLP was also found to associate with BMD [9] the way that „BB" genotype was preponderant in patients with low BMD. Repeat studies including diverse ethnic groups were controversial. Some confirmed these findings while others could not demonstrate an association or attributed vice versa the „b" alleles to low BMD [10,39,40]. This was also true when extending the studies to other allelic variants. It is noteworthy that none of the polymorphisms is associated with sequence alterations of the VDR gene. They are either in the non-coding region or are neutral variants coding for the same aminoacid (e.g., isoleucin, exon 9 polymorphism). Nevertheless there are a variety of explanations for these discrepant findings. In the region of intron 8 to exon 9 (*Bsm*I, *Apa*I, and *Taq*I RFLPs), and exon 2 (*Fok*I RFLP) there may be a decrease in BMD with the B, and also A, t, and f alleles but the intergroup differences and the number of individuals could be too small to be detected by statistics. All these markers are only biallelic reflecting three genotypes which needs a high statistical power to evaluate particular association. Some studies are done in populations with different or varying ethnicities for which e.g., the *Bsm*I RFLP might not be informative in all populations (Table 6).

A major question is how allelic differences might relate to functional differences, as they are not in the VDR coding region. They can be just neutral polymorphisms that can serve as genetic markers for a nearby modifying gene or they can directly influence or just be in linkage disequilibrium with sequence elements that affect mRNA. There are only a few studies concentrating to the possible mechanisms by which the polymorphisms might function. In case of the *Fok*I polymorphism a functional relation is suggestive. A transition in exon 2 (C→T) creates a second start codon (ACG→ATG) three codons upstream. This might cause a change in the predicted protein sequence [12]. In a reporter gene assay the larger VDR variant was less efficient [41]. In case of BB and bb fibroblast cells no difference in the expression of both alleles was observed, indicating that the *Bsm*I RFLP is not functionally related but rather a genetic marker for other variants of the same or another adjacent gene [42]. This is further supported by a variety of other positive correlations e.g., to risk of osteoarthritis [36], hyperthyroidism [37], and prostate cancer [38].

The functional relevance of the Spl polymorphism may contribute to the severity of Ol. We postulate a hypothesis, where the clinical outcome of Ol or osteoporosis may be attributed to a mutation, whether in the *COLIA1*, *COL1A2* genes or not, and to the nature of the Spl polymorphism (Table 7; Figure 3).

**Figure 3** Synopsis of possible constellations of collagen type I mutations and Sp1 polymorpisms. Predictions of genotype-phenotype correlations are given in Table 7.

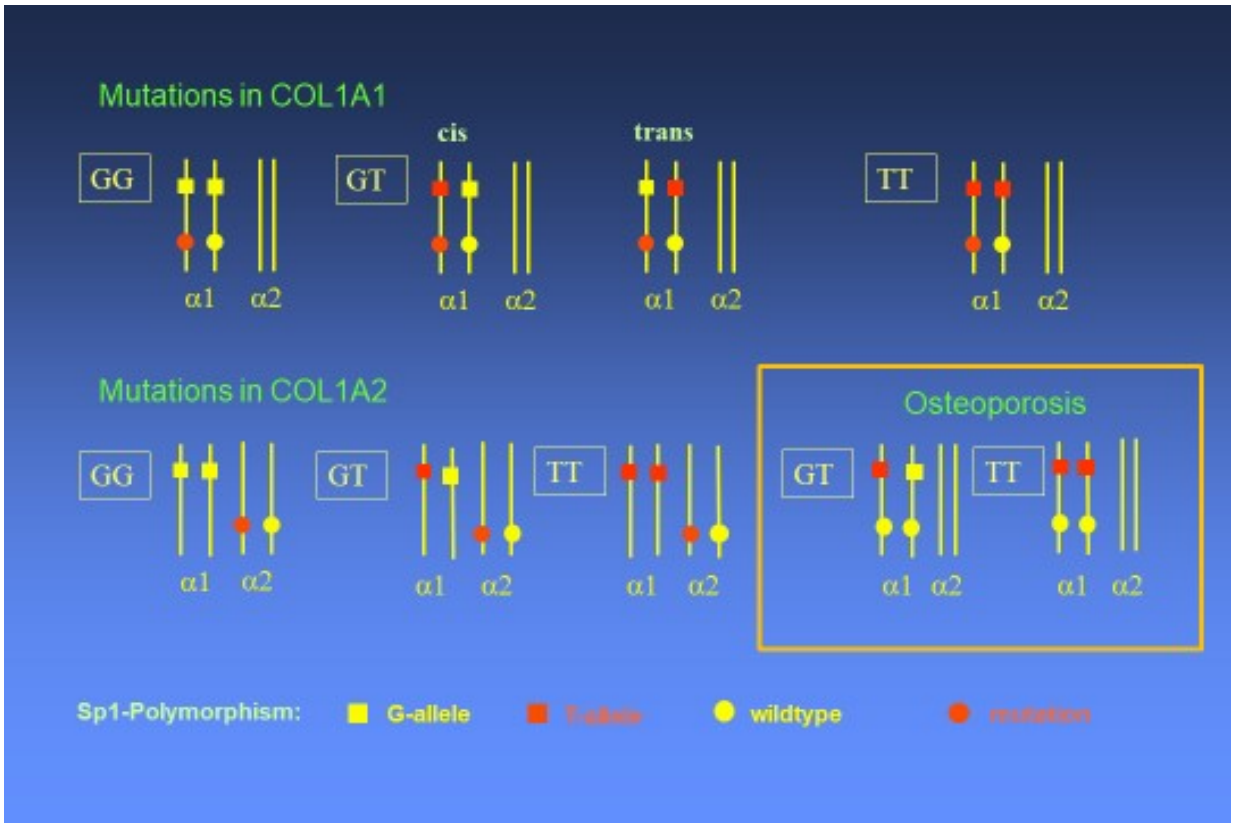


**Table 7** Predictions of the clinical outcome of Ol and risk of osteoporosis depending on the Spl polymorphism relative to putative mutations in the *COL1A1* and *COL1A*2 genes.

| COL1A1 | | | COL1A2 | Phenotype | Effect of Sp1 Polymorphism |
|---|---|---|---|---|---|
| mutant **y**es/**n**o | genotype | transcription levels of relevant (i.e. mutant and/or T) allele(s) | mutant **y**es/**n**o | | |
| n | G/G | none | n | healthy | none |
| n | T/T | high | n | risk for osteoporosis | putative homotrimer formation |
| n | G/T | moderate | n | healthy | none |
| **y** | G/G | low | n | Ol mild to moderate | none |
| **y** | T/T | high | n | Ol severe | increased incorporation of mutant allele |
| **y** | G/T, mutation and polymorphism in cis | high | n | Ol moderate to severe | abundant mutant T fibers compete with wildtype G fibers |
| **y** | G/T, mutation and polymorphism in trans | low | n | Ol mild | abundant wildtype T fibers compete with mutant G fibers |
| n | G/G | low | y | Ol moderate to severe | depends on the nature of the mutation. Low frequency of α1(I) trimer formation |
| n | T/T | high | y | Ol mild to moderate | depends on the nature of the mutation. high frequency of αl(I) trimer formation |
| n | G/T | moderate | y | OI moderate | depends on the nature of the mutation. Moderate frequency of α1(I) trimer formation |

Depending on the position of a *COLIAI* mutation in cis or trans to the polymorphism, the phenotype of Ol may be mild or more severe (Figure 3). Thus, an identical *COLIAI* mutation may lead to different types of Ol, depending on the Spl polymorphism. This hypothesis is due to the triple-helical nature of the collagen fiber. There are always two αl(I) and one α2(I) proteins in the helix. If the disease-causing mutation is in *cis* with the abundantly transcribed T allele, there will also be an over-representation of incorporated mutant proteins, leading to an aggravation. However, if the T allele is on the wildtype allele, then the wildtype allele will be transcribed at a higher rate and, thus, will compete with the mutant allele during helix

formation. The increased incorporation of the wildtype allele will help to mitigate the clinical manifestation of OI.

Likewise, one could easily imagine that the increased T allele-specific levels may also influence the assembly of the helix even if the mutation affects the α2(I) helix. The absence of intact α2(I) fibers due to a deleterious mutation may promote the formation of homotrimeric αl(I) helices. C-terminal truncations of the α2(I) fiber are the archetype for αl(I) homotrimer formation. By means of an increased number of homotrimers of the T-specific transcript of the wildtype αl(I), mutant α2(I) fibers may not join the helix and the phenotype will be less severe. Our hypothesis confirms recent data on the Spl polymorphism in patients with osteoporosis. The T allele will increase the incorporation of αl(I) fibers which will lead to a reduced BMD and reduced stability of bone. Our hypothesis is based on threshold transcript/protein levels, where minute dosage differences may contribute to the severity of the phenotype. Therefore, Ol is another example of an apparently monogenic disorder with considerable clinical variance, where genetic modifiers are of importance. An increasing number of inborn errors of metabolism with threshold values and genetic modifiers will come up in the future as it was already predicted in an editorial note [43].


## Acknowledgment

The skillful technical assistance of Alexandra Killian is greatly appreciated.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