Gene-Wide Association Study of *RANK* and *RANKL* genes in the Bone Context: Functional Study of BMD-associated SNPs

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Introduction

Over the past decade, many GWAs and meta-analyses were performed to identify genes and regions involved in bone metabolism and in the osteoporotic phenotypes. Nevertheless, the majority of these GWAS results were not tested at any functional level. This study aims to find and study functional regions in the *Receptor Activator of Nuclear Factor kappa-B (RANK) and its ligand (RANKL)* genes that encode well-established proteins in the bone remodeling equilibrium.

Methods

Thirty-three SNPs, chosen for their location in an evolutionary conserved region or replicated from previous studies, (Figure 1) were genotyped in the BARCOS cohort of 1061 postmenopausal women (Table 1).

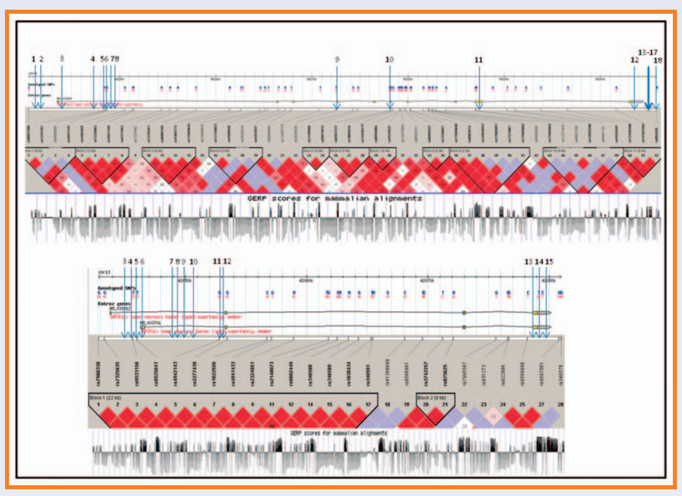

Fig. 1: Genotyped SNPs in the *RANK* (above) and *RANKL* (below) genes. Each SNP is represented by its corresponding number in Table 2. For each gene, its chromosomal location, the HapMap haplotypic blocks (Haploview software) and the evolutionary conserved profile (UCSC genome browser) are shown. Note that *RANKL* SNPs rs9594738 and rs9594759 (numbered 1 and 2 in Table 2) do not appear in this figure due to their far upstream position.

Table 1. Baseline characteristics of the BARCOS cohort

Patients characteristics	Phase 1		Phase 2	
	Mean ± SD	n	Mean ± SD	n
Age at menopause (years)	48.27 ± 3.92	884	48.41 ± 3.91	1061
BMI	26.35 ± 3.87	884	26.15 ± 3.84	1058
Breastfeeding (months)	7.97 ± 13.24	884	7.79 ± 12.82	1054
Age at LS densitometry (years)	55.64 ± 8.55	884	55.98 ± 8.44	1057
Years since menopause LS	7.37 ± 8.35	884	7.59 ± 8.27	1057
LS BMD (g/cm^2)	0.853 ± 0.15	884	0.854 ± 0.15	1060
Age at FN densitometry (years)	57.71 ± 8.03	801	57.74 ± 7.95	972
Years since menopause FN	9.41 ± 7.90	801	9.30 ± 7.87	972
FN BMD (g/cm^2)	0.683 ± 0.11	801	0.684 ± 0.11	976
Menarche age (years)	12.91 ± 1.60	874	12.88 ± 1.60	1044
Fractures	135 (15.3%)	884	145 (13.7%)	1061

Results

SNP rs9594738, which lies 184 bp upstream of the *RANKL* gene, was found to be associated with lumbar spine bone mineral density (Log additive model: beta coefficient= -0.021, p=3.8x10^{-4}) (Table 2). Functional experiments exploring this *RANKL* distal region (DR) harboring rs9594738 demonstrated the region's capacity to inhibit the *RANKL* promoter in reporter gene assays (Figure 2). Moreover, DR was activated in vitamin D presence (Figure 3).

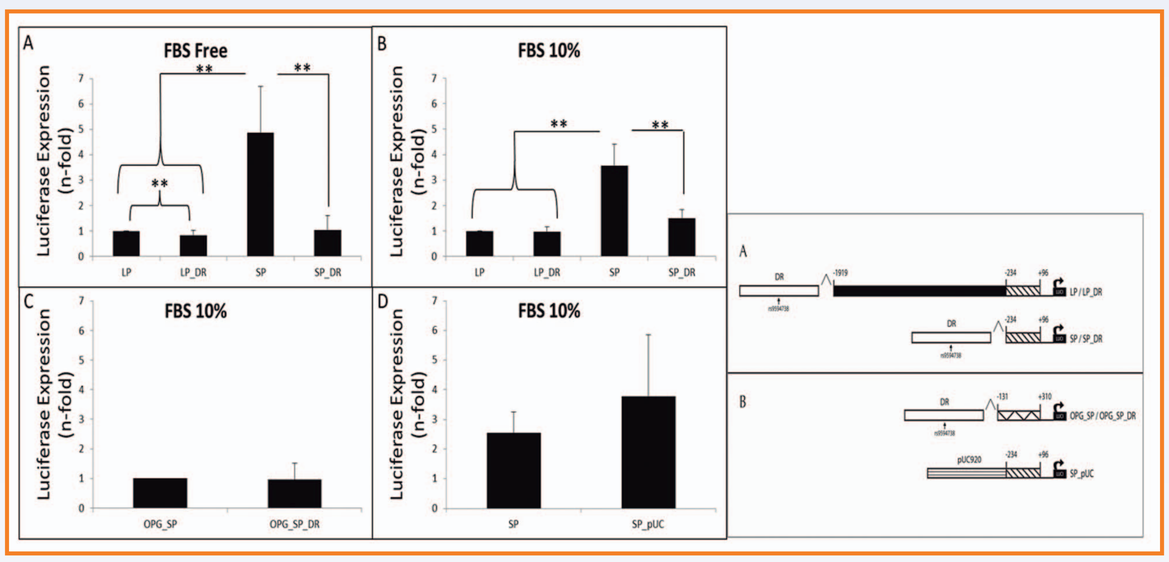

Fig. 2. Reporter gene assays. Results are means (± SD). For A, B and D, each construct was compared to LP, which was arbitrarily set at 1. (A) The *RANKL* LP and SP promoter constructs, with and without DR, were tested after overnight incubation in DMEM + 0.1% BSA. The number of replicates was n = 20. (B) The *RANKL* LP and SP promoter constructs, with and without DR, were tested after overnight incubation in DMEM + 10% FBS. The number of replicates was n = 9. (C) The *OPG* basal promoter, with and without the DR segment, tested after overnight incubation in DMEM + 10% FBS. The number of replicates was n = 5. OPG_SP_DR was compared to OPG_SP, which was arbitrarily set at 1. (D) SP construct with an additional 920 bp segment from pUC19 compared to SP, tested after overnight incubation in DMEM + 10% FBS. The number of replicates was n = 6. **p < 0.01, Wilcoxon test.

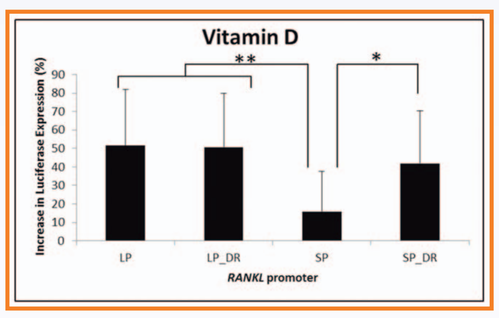

Fig. 3. Reporter gene assay in cells treated with vitamin D. Results are means (± SD) of increased (%) luciferase activity compared to non treated cells. The number of replicates was n = 12. *p < 0.05; **p < 0.01, Wilcoxon test.

Table 2. List of the genotyped gene-wide SNPs, genotyping efficiency, MAF and p-values for log-additive model

Gene	SNP #	rs	location	n	efficiency	MAF BARCOS	HWE	LS	FN	Fractures
RANKL	1	rs9594738	184kb upstream	1061	0.97	0.44	0.80	3.4x10^{-4} (1.5x10^{-4})^{d}	0.07 (0.02)^{d}	0.56
	2	rs9594759	104kb upstream	884	0.87	0.49	0.15	0.13	0.81	0.37
	3	rs17639305	Proximal promoter	884	0.92	0.17	0.38	0.63	0.86	0.39
	4	rs9533156	Proximal promoter	884	0.89	0.46	0.34	0.53	0.97	0.16
	5	rs9525641	Proximal promoter	884	0.91	0.45	0.24	0.64	0.96	0.18
	6	rs2296533	Exon 1	884	0.76	0.46	0.21	0.72	0.61	0.24
	7	rs9594782	Intron 1	884	0.92	0.07	0.39	0.86	0.82	0.49
	8	rs12427596	Intron 1	884	0.91	0.43	0.49	0.94	0.77	0.10
	9	rs9525642	Intron 1	884	0.92	0.38	0.99	0.87	0.97	0.03
	10	rs9533158	Intron 1	884	0.86	0.16	0.84	0.64	0.99	0.48
	11	rs9533159	Intron 1	884	0.90	0.43	0.24	0.85	0.67	0.10
	12	rs2277438	Intron 1	884	0.92	0.17	0.50	0.77	0.87	0.30
	13	rs9562415[1]	Exon 5	1061	0.95	0.02	0.55	0.65	0.71	0.28
	14	rs9567000[1]	3' UTR	1061	0.97	0.02	0.57	0.77	0.50	0.08
	15	rs1054016	3' UTR	884	0.87	0.40	0.56	0.73	0.74	0.15
Gene	SNP #	rs	location	n	efficiency	MAF BARCOS	HWE	LS	FN	Fractures
RANK	1	rs6567265	Proximal promoter	884	0.83	0.30	0.38	0.66	0.85	0.25
	2	rs7233419	Proximal promoter	884	0.90	0.30	0.17	0.69	0.97	0.77
	3	rs12457042	Intron 1	884	0.92	0.06	0.56	0.09	0.66	0.29
	4	rs11152341	Intron 1	884	0.89	0.24	0.30	0.14 (0.037)^{o}	0.85	0.50
	5	rs7233197	Intron 1	884	0.93	0.07	0.54	0.10	0.71	0.07
	6	rs4941125	Intron 1	884	0.92	0.29	0.20	0.32	0.36	0.72
	7	rs4941126	Intron 1	884	0.89	0.28	0.32	0.36	0.40	0.83
	8	rs12150741	Intron 1	1061	0.97	0.24	0.13	0.27 (0.029)^{o}	0.59	0.49 (0.035)^{r}
	9	rs35211496	Exon 4[2]	884	0.83	0.22	0.93	0.55	0.45	0.48
	10	rs1805034	Exon 6[3]	1061	0.97	0.42	0.44	0.73	0.57	0.15 (0.049)^{d}
	11	rs8092336[1]	Exon 9	884	0.93	0.04	0.22	0.86	0.63	0.90
	12	rs78622775[1]	3' UTR	1061	0.98	0.01	0.73	0.30	0.34	0.99
	13	rs12455323	3' Region	1061	0.97	0.32	0.89	0.79	0.92	0.86
	14	rs72933640	3' Region	1061	0.97	0.13	0.006	0.19	0.93	0.58
	15	rs78326403	3' Region	1061	0.97	0.08	0.28	0.77	0.43	0.05 (0.021)^{o}
	16	rs78459945	3' Region	1061	0.97	0.08	0.40	0.97	0.34	0.18 (0.035)^{o}
	17	rs72933641	3' Region	1061	0.97	0.13	0.07	0.27	0.89	0.56
	18	rs884205	3' Region	1061	0.97	0.19	0.36	0.59	0.69	0.07 (0.0087)^{r}

[1]Due to low MAF for rs9562415, rs956700, rs8092336 and 78622775 the only available statistical model was the codominant model. For the rest of the SNPs, in case of lower significant p value in other model rather than log-additive, the results are given in (). [2]p.H141Y. [3]p.A192V. MAF=minor allele frequency; HWE=Hardy Weinberg equilibrium; LS=lumbar spine; FN=femoral neck; Bold p< 0.05; [d]Dominant. [o]Overdominant. [r]Recessive.

Conclusions

Our results demonstrate DR functionality in the *RANKL* gene context, with a vitamin D involvement.

Disclosures

The authors state that they have no conflicts of interest.