A Functional SNP in *BNC2* Is Associated with Adolescent Idiopathic Scoliosis

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Adolescent idiopathic scoliosis (AIS) is the most common spinal deformity. We previously conducted a genome-wide association study (GWAS) and detected two loci associated with AIS. To identify additional loci, we extended our GWAS by increasing the number of cohorts (2,109 affected subjects and 11,140 control subjects in total) and conducting a whole-genome imputation. Through the extended GWAS and replication studies using independent Japanese and Chinese populations, we identified a susceptibility locus on chromosome 9p22.2 ($p = 2.46 \times 10^{-13}$; odds ratio = 1.21). The most significantly associated SNPs were in intron 3 of *BNC2*, which encodes a zinc finger transcription factor, basonuclin-2. Expression quantitative trait loci data suggested that the associated SNPs have the potential to regulate the *BNC2* transcriptional activity and that the susceptibility alleles increase *BNC2* expression. We identified a functional SNP, rs10738445 in *BNC2*, whose susceptibility allele showed both higher binding to a transcription factor, YY1 (yin and yang 1), and higher *BNC2* enhancer activity than the non-susceptibility allele. *BNC2* overexpression produced body curvature in developing zebrafish in a gene-dosage-dependent manner. Our results suggest that increased *BNC2* expression is implicated in the etiology of AIS.

Adolescent idiopathic scoliosis (AIS) is defined by lateral spinal curvature with a Cobb angle of at least 10° that occurs in otherwise healthy children from the age of 10 to the end of the pubertal growth spurt.[1] AIS affects 2%–3% of adolescents worldwide.[1] Female preponderance of AIS has been described;[2] the prevalence of AIS in females with Cobb angles >10° and >20° is 11 and 20 times higher, respectively, than that in males with Cobb angles >10° and >20°.[2] A multifactorial etiology with a polygenic component has been suggested.[3,4] The heritability of AIS is estimated as 87.5%, and is higher for females than for males, in a previous study examining first-degree relatives.[5] A female-specific gene associated with AIS, *PAX1* (MIM: 167411), has been reported recently.[6]

We previously conducted a genome-wide association study (GWAS) of Japanese females (1,033 affected subjects and 1,473 control subjects) and identified two loci on chromosomes 10q24.31[7] and 6q24.1.[8] The association of these loci with AIS was replicated in Chinese and Caucasian populations;[9–11] however, these loci only explain ~1% of the total genetic variance in AIS.[8]

To identify additional susceptibility gene(s) for AIS, we extended our GWAS by increasing the numbers of subjects (Table S1) and conducting a whole-genome imputation. A total of 2,142 AIS-affected subjects were recruited for the GWAS from ten collaborating hospitals (Japanese Scoliosis Clinical Research Group), according to the criteria previously described.[7,8,12] All underwent clinical and radiologic examinations by expert scoliosis surgeons. 11,144 control subjects for the GWAS were drawn from the BioBank Japan Project (see Web Resources) and its related projects, as previously described.[13–15] The study was approved by the institutional review boards of RIKEN and participating hospitals. A written informed consent was obtained from all participants and/or guardians on the behalf of the minors and/or children participants.

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http://dx.doi.org/10.1016/j.ajhg.2015.06.012.

Table 1. Association of rs3904778 with Adolescent Idiopathic Scoliosis

Population	Study	Number of Samples		RAF		p Value[a]	Odds Ratio (95% CI)
		Affected	Control	Affected	Control		
Japanese	GWAS	2,109	11,140	0.459	0.414	2.10×10^{-7}	1.20 (1.12–1.28)
	replication	955	3,551	0.476	0.424	4.46×10^{-5}	1.23 (1.12–1.37)
	Japanese combined	3,064	14,691	–	–	5.08×10^{-11}	1.21 (1.14–1.28)
Chinese	replication	1,268	1,173	0.429	0.384	1.14×10^{-3}	1.20 (1.07–1.35)
All[b]	all combined	4,332	15,864	–	–	2.46×10^{-13}	1.21 (1.15–1.27)

Abbreviations are as follows: RAF, risk-allele frequency; CI, confidence interval.
[a]Cockran-Armitage trend test.
[b]Japanese and Chinese.

We genotyped 1,289 AIS-affected subjects and 3,345 control subjects by using the Illumina Human610 Genotyping BeadChip and Illumina HumanHap550v3 Genotyping BeadChip and 853 AIS-affected subjects and 7,799 control subjects by using the Illumina Human OmniExpress Genotyping BeadChip and Illumina Human Exome Genotyping BeadChip (Table S1). Quality-control measures have been described previously.[7] For quality control of the samples, we checked gender information by using SNPs on the X chromosome and evaluated cryptic relatedness for each sample with the identity-by-state method and removed samples that showed second-degree relatedness or closer. To check population stratification of this study, we performed principal-component analysis (PCA) with four reference populations from the HapMap data: CEU (Utah residents with ancestry from northern and western Europe from the CEPH collection), YRI (Yoruba in Ibadan, Nigeria), JPT (Japanese from Tokyo, Japan), and CHB (Han Chinese from Beijing, China) (Figures S1A and S1B). We plotted the scatter plot by using the top two associated principal components (eigenvectors) to identify outliers who did not belong to the JPT and CHB cluster. Subsequently, we performed PCA by using only the genotype information of the affected and control subjects to further evaluate the population substructure (Figures S1C and S1D). After quality-control filtering, 458,596 SNPs in the Illumina Human610 Genotyping BeadChip and 605,478 SNPs in the Illumina Human OmniExpress Genotyping BeadChip were examined for association. We checked for possible population-stratification effects by constructing a quantile-quantile (Q-Q) plot using observed p values against expected p values (Figure S2) and obtained genomic inflation factors (λ-value) of 1.06 and 0.97 in each platform, respectively, implying that the possibility of false-positive associations due to population structure or cryptic relatedness was low.

Because the tag SNPs involved in the two platforms were different, we performed a whole-genome imputation and a meta-analysis. In brief, we used the 1000 Genomes Integrated Phase I release version 2 dataset of East Asian populations (JPT, CHB, and CHS [Southern Han Chinese]) as a reference panel to infer missing genotypes. After quality control, we prepared the input files, which excluded SNPs with a genotyping rate of <99% and a MAF of <0.01 and those that deviated from Hardy-Weinberg equilibrium ($p \leq 1.0 \times 10^{-6}$). We also excluded SNPs whose allele frequencies had differences of >0.16 between the GWAS dataset and the reference panel. We used SNPs with an imputation quality score Rsq (R-square) ≥ 0.9 for the association study. The associations of the imputed SNPs were generated with mach2dat software, which utilized the output results from Minimac. Combined meta-analysis of the discovery and validation phases was performed with the inverse-variance method; heterogeneity between the two phases was evaluated by the Breslow-Day test, and SNPs with a p value < 0.05 were removed. In total, we analyzed the association of 4,420,789 SNPs for 2,109 Japanese subjects with AIS and for 11,140 control subjects. Three loci surpassed the genome-wide significance level of $p < 5 \times 10^{-8}$; the two previously identified loci on 10q24.31[7] and 6q24.1[8] topped the list (Table S2).

To confirm the association of the loci, we recruited an independent set of 958 AIS-affected subjects from the collaborating hospitals and 3,551 control subjects (Table S1) and conducted a replication study. We selected 27 SNPs that surpassed $p < 1.0 \times 10^{-5}$ in the GWAS (Table S2) and genotyped the subjects with a multiplex PCR–based Invader assay (Third Wave Technologies), as previously described.[8] In the replication study, we found evidence for association with one locus represented by the SNP, rs3904778, on chromosome 9p22.2 and with a p value of 4.46×10^{-5} (Bonferroni-corrected $p < 1.85 \times 10^{-3}$; Table 1). When the results of the GWAS and the replication study were combined, rs3904778 reached a genome-wide significance threshold of $p < 5 \times 10^{-8}$ (combined $p = 5.08 \times 10^{-11}$; odds ratio [OR] = 1.21, 95% confidence interval = 1.14–1.28; Table 1).

To further test this association, we genotyped rs3904778 in a Han Chinese population recruited by the Nanjing Drum Tower Hospital, affiliated with the Nanjing University Medical School. AIS-affected subjects were diagnosed through clinical and radiological examinations according to the previously described criteria.[8] The control subjects were healthy volunteers whose absence of scoliosis was

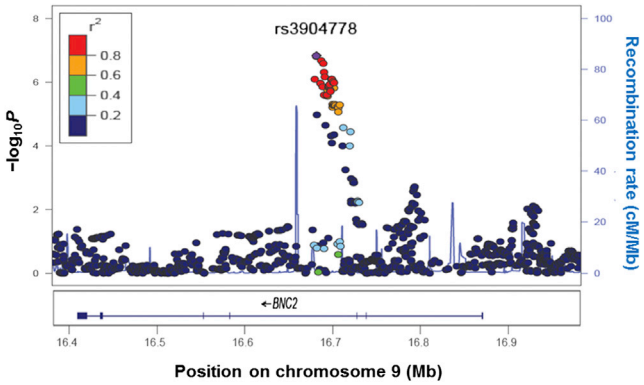

Figure 1. Regional Association Plot for the *BNC2* Locus on 9p22
The $-\log_{10}$ (p value) of SNP association with adolescent idiopathic scoliosis was plotted against the chromosome positions (NCBI build 37) with LocusZoom software. The recombination rate estimate (blue line) is based on East Asian genotype data from 1000 Genomes. The SNP with the highest association signal (rs3904778) is represented by a purple circle. Other SNPs are colored according to their linkage disequilibrium (r^2) with rs3904778. The most significantly associated SNPs are clustered in intron 3 of *BNC2*.

confirmed through the Adam's forward bend test by an experienced spinal surgeon of the hospital (Y.Q.; Table S1). We genotyped 1,268 affected subjects and 1,173 control subjects by using a TaqMan SNP Genotyping Assay read with an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The association was replicated in the Chinese population. The p value of the overall meta-analysis was 2.46×10^{-13} and the OR was 1.21 (Table 1).

To characterize the locus on chromosome 9p22.2, we plotted, genotyped, and imputed SNPs around rs3904778 (Figure 1). Three additional SNPs yielded evidence for association and were strongly correlated ($r^2 \geq 0.9$) with rs3904778 (Table S3). All of these SNPs were located in intron 3 of *BNC2* (basonuclin-2 [MIM: 608669]; Figure 1).

BNC2 is a highly conserved protein that belongs to the group of C2H2 zinc finger proteins. Mouse BNC2 has 97.0% amino-acid identity with human BNC2, and zebrafish Bnc2 has 60.8% identity.[16] BNC2 is concentrated in the nuclear speckles, suggesting a function in nuclear processing of mRNA.[17] In mice, *Bnc2* expression has been observed in the craniofacial bones, ovary, uterus, and brain.[18–20] In zebrafish, *bnc2* expression has been observed in the ovary and CNS, namely the sensory ganglia, hindbrain, and eyes.[21] However, *BNC2* expression in human musculoskeletal tissues had not previously been explored. Therefore, we examined the expression of *BNC2* mRNA by using qRT-PCR in various human tissues (Table S4). We found that *BNC2* was most highly expressed in the uterus and spinal cord, followed by bone and cartilage (Figure S4).

We investigated possible functional effects of the associated SNPs by cross-referencing expression quantitative trait locus (eQTL) data for the locus of chromosome 9p22.2 with the Genevar (Gene Expression Variation) database.[22] Only rs10738445 was found in the database of fibroblast experiments, and its susceptibility allele significantly increased *BNC2* expression ($p = 0.048$ [Mexican population in Genevar]). We also evaluated the overlap of associated SNPs with Encyclopedia of DNA Elements (ENCODE)-annotated genomic elements by using the UCSC Genome Browser. Only rs3850444 was located within DNase-I-hypersensitive regions across multiple tissues. Three SNPs had enhancer histone marks: rs3850444 and rs3904778 in H1 embryonic stem cells and NHLFs (normal human-lung fibroblasts) and rs10738445 in human skeletal-muscle myoblasts and NHLFs. These findings suggest that the SNPs have the potential to regulate the transcriptional activity of *BNC2*.

We examined the transcriptional activity of the SNPs by using luciferase assays. We constructed reporter vectors by cloning oligonucleotides around each SNP (Table S5) into a pGL3 promoter vector (Promega). HeLa cells were transfected with the reporter vectors and pRL-TK vector (Promega) by a FuGene6 transfection reagent (Promega), according to the manufacturer's protocol. After 48 hr incubation, we compared the luciferase activities between the constructs with risk alleles and those with non-risk alleles. Only rs10738445 had enhancer activity and significant allelic difference. The construct containing the risk allele showed approximately 1.3-fold higher luciferase activity than the construct containing the non-risk allele (Figure 2A), suggesting that the risk allele of rs10738445 is implicated in AIS by increasing the sequences' enhancer activity. Experiments using other cell lines (HEK293 and A172) yielded similar results (data not shown).

To evaluate whether the allelic difference had an effect on rs10738445 binding, we performed an electrophoretic mobility shift assay (EMSA) for nuclear extracts from HeLa cells by using the DIG (digoxigenin) Gel Shift Kit, 2nd generation (Roche) and double-stranded oligonucleotides (Table S5). A specific band to the oligonucleotide containing the risk allele was detected (Figure 2B). Using the JASPAR database, we looked for a transcription factor that might have altered binding.[23] We found that rs10738445 is located within a YY1 binding site and speculated that it might have altered binding. We performed an EMSA by using a YY1 antibody (Santa Cruz). The band specific to the risk-allele disappeared and shifted with the addition of an antibody against YY1 (Figure 2B). We next examined the effects of YY1 on rs10738445 by using a luciferase assay. We constructed a YY1 expression vector by cloning the coding sequence into pcDNA3.1(+). We co-transfected rs10738445 reporter plasmids and YY1 expression plasmids or empty pcDNA3.1(+) to HeLa cells. The YY1 expression vector significantly increased the luciferase activity with significant allelic difference (Figure 2C).

To examine in vivo effects of *BNC2* overexpression, we utilized the tol2-mediated transgenesis system[24,25] in zebrafish. We PCR-amplified the *BNC2* coding region

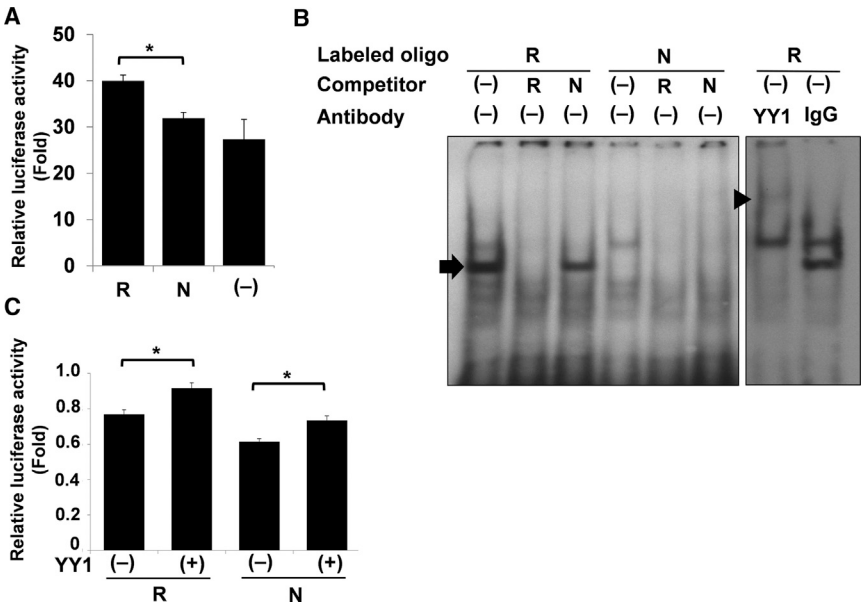

Figure 2. Allelic Difference of rs10738445 Function In Vitro

(A) Transcriptional enhancer activities of rs10738445 constructs and pGL3 promoter vector (−). The construct containing risk (susceptibility)-allele (R) showed approximately 1.3-fold higher luciferase activity than the construct containing non-risk allele (N). *p value < 0.01 (t test). Error bar: standard error. The experiment was repeated three times.

(B) Electrophoretic mobility shift assay. Unlabelled probes (a 200-fold excess) were used as competitors. Addition of an antibody against YY1 resulted in the disappearance of a risk-allele-specific band (arrow) and supershift (arrowhead).

(C)Transcriptional enhancer activities of rs10738445 constructs with and without a YY1 expression vector. Addition of YY1 increased the luciferase activity in both R and N. The luciferase activity was higher in the construct containing R than in that containing N. *p value < 0.05 (t test). Error bar: standard error.

from a cDNA clone (IMAGE: 100069207) by using specific primers attached to restriction-enzyme sequences (Table S4). To generate *Tol2* transgenesis constructs, *p5E-bactin2*, *pME-MCS BNC2* clone, and *p3E-polyA* were recombined into *pDest-Tol2pA2* by Gateway LR Clonase II enzyme mix (Life Technologies). We co-injected plasmid DNA (10 ng/μl) into one-cell-stage embryos of the RIKEN wild-type strain (*Danio rerio*, provided by the National BioResource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan) with capped *Tol2* transposase mRNA (50 ng/μl) that was synthesized with a mMESSAGE mMACHINE SP6 Kit (Ambion). We stably expressed *BNC2* in zebrafish embryos and analyzed founder transgenic embryos between 24 and 72 hr post-fertilization (hpf). Overexpression of *BNC2* in zebrafish embryos resulted in body curvature to various degrees (65%, n = 150) and embryonic lethality (18%, n = 41), whereas EGFP-overexpressing control embryos underwent normal development (Figures 3A and 3B). Most of the abnormal embryos that were delivered with the *BNC2* transgene exhibited severe body curvature, and some displayed malformation of the somite, resulting in larval death within one week.

We next injected *BNC2* mRNA into the one-cell-stage embryos to analyze phenotypes at various doses. We cloned the *BNC2* coding sequence into pcDNA3.1, synthesized capped mRNA, and injected it at doses of 10–200 pg into the one-cell-stage embryos. Increasing doses of *BNC2* mRNA were correlated with the severity of embryonic body curvature (Figures 3C and 3D). Abnormal somite formation of *BNC2*-overexpressing embryos was evident from somite stages 13 to 20 (Figure S5). *BNC2*-overexpressing embryos exhibited axial body curvature (Figure 3) at 24 hpf and also lacked a normal segmental expression pattern of *myod*, which was expressed in the myotome occupying a large part of the zebrafish somites (Figure S6). At higher doses, delayed or disturbed pigmentation was also observed in the embryos with severe body curvature (Figure 3C, arrowheads). These results suggest that increased expression of *BNC2* leads to embryonic body curvature, which is likely to be relevant to scoliosis.

The mechanism behind how BNC2 causes AIS remains to be examined. Neuropathic abnormality and abnormal musculoskeletal growth have been implicated in the etiology of AIS. *BNC2* could play a role under either hypothesis. We found that *BNC2* is highly expressed in the spinal cord, bone, cartilage, and, to a lesser extent, human muscle. Mouse *Bnc2* is also observed in the CNS and craniofacial bones, and the knock-out mice are small with craniofacial abnormalities.[18–20] Knock-down zebrafish also exhibit small size, suggesting a role of *bnc2* in skeletal development.[21] Scoliosis was not observed in the knock-down mice or zebrafish. We overexpressed *bnc2* and observed embryonic body curvature in zebrafish. Body curvature at the early stages later develops into scoliosis in *skolios,* a recessive mutant with a nonsense mutation in kinesin family member 6.[26] Further studies are necessary to examine whether scoliosis caused by increased *BNC2* is due to neuronal and/or musculoskeletal (bone, cartilage, and muscle) abnormality.

In summary, through an extended GWAS using ~13,000 Japanese subjects, we have identified a AIS-susceptibility gene, *BNC2*. The association of the most significant SNP in the gene was replicated in independent Japanese and Han-Chinese populations, and the overall p value was 2.46×10^{-13}. The highly associated SNPs were all in intron 3 of *BNC2*. eQTL data for the locus suggested both that the associated SNPs have the potential to regulate the transcription of *BNC2* and that the susceptibility alleles have the potential to significantly increase *BNC2* expression. In vitro studies showed that a genomic region containing one of the significantly associated SNPs, rs10738445, had

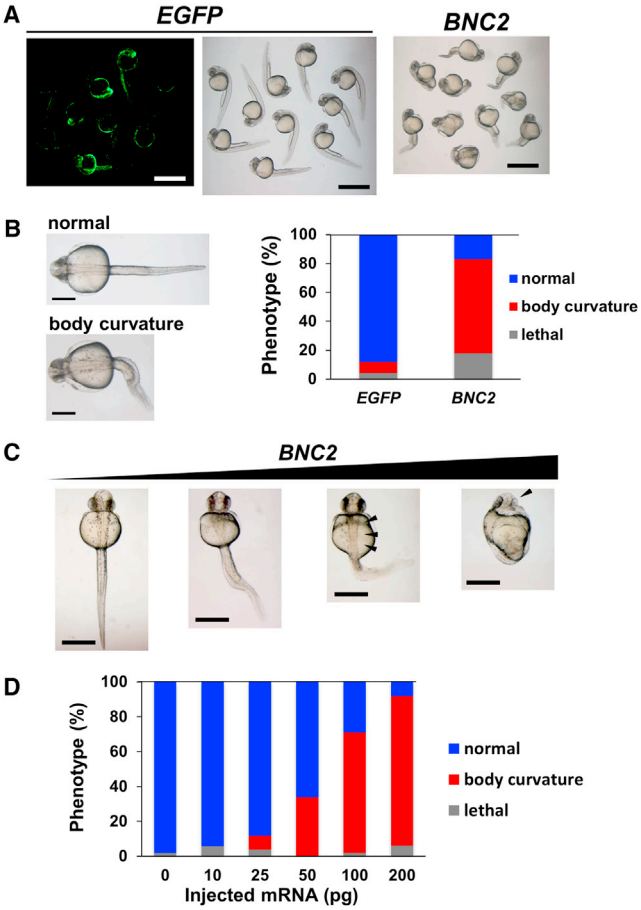

Figure 3. Overexpression of *BNC2* in Zebrafish Embryos
(A) Embryos (24 hpf) overexpressing *EGFP* (control) and *BNC2*. Transgenes were stably expressed by tol2-mediated transgenesis. Ubiquitous transgene expression was confirmed by green fluorescence in control founder embryos (left). The *BNC2* transgenic embryos exhibited severe body curvature and malformation of the somite. Scale bars, 1 mm.
(B) Quantification of developmental phenotypes of embryos overexpressing *EGFP* (control) or *BNC2*. The percentages of the number of embryos that died by 24 hpf (lethal), had deformed somites and body curvature (body curvature), or had no apparent abnormality in the somites (normal), are shown along with the representative images of embryos exhibiting the "normal" or "body curvature" phenotypes. Scale bars, 300 μm.
(C) Zebrafish embryos (24 hpf) injected with increasing doses of *BNC2* mRNA. The mRNA injection caused body curvature and severe malformation of the somites in a dose-dependent manner. Arrowheads indicate delayed pigmentation of the embryo injected with the mRNA at the higher doses (100 and 200 pg). Scale bars, 500 μm.
(D) Quantification of developmental phenotypes of embryos injected with BNC2 mRNA. Embryos between 24 and 28 hpf were evaluated morphologically as in (B).

an enhancer activity, which is stronger in the disease-associated allele. YY1 binds to the genomic region containing rs10738445 and upregulates *BNC2* expression. Both binding and upregulation were stronger in the disease-associated allele of rs10738445. The zebrafish experiments supported the hypothesis that increased *BNC2* expression predisposes individuals to AIS.

Supplemental Data

Supplemental Data include six figures and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.ajhg.2015.06.012.

Acknowledgments

We are grateful to the individuals who participated in this study. We thank N. Suzuki, M. Saito, M. Kamata, and N. Fujita for their help in collecting the samples. We also thank T. Isono, S. Tominaga, Y. Takanashi, and T. Kusadokoro for technical assistance and N. Atsumi and S. Unger for English language editing.

Received: April 15, 2015
Accepted: June 26, 2015
Published: July 23, 2015

Web Resources

The URLs for data presented herein are as follows:

1000 Genomes, http://www.1000genomes.org/
BioBank Japan Project, http://biobankjp.org/
ENCODE, http://genome.ucsc.edu/ENCODE/
Genevar (Gene Expression Variation), http://www.sanger.ac.uk/resources/software/genevar/
JASPAR, http://jaspar.genereg.net/
LocusZoom, http://locuszoom.sph.umich.edu/locuszoom/
MACH 1.0, http://www.sph.umich.edu/csg/abecasis/MACH/index.html
Minimac, http://genome.sph.umich.edu/wiki/Minimac
OMIM, http://www.omim.org/
The R Project for Statistical Computing, R v.2.13.0, http://www.r-project.org/
UCSC Genome Browser, http://genome.ucsc.edu

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