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A variant associated with sagittal nonsyndromic craniosynostosis alters the regulatory function of a non-coding element

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Craniosynostosis presents either as a nonsyndromic congenital anomaly or as a finding in nearly 200 genetic syndromes. Our previous genome-wide association study of sagittal nonsyndromic craniosynostosis identified associations with variants downstream from *BMP2* and intronic in *BBS9*. Because no coding variants in *BMP2* were identified, we hypothesized that conserved non-coding regulatory elements may alter *BMP2* expression. In order to identify and characterize noncoding regulatory elements near *BMP2*, two conserved noncoding regions near the associated region on chromosome 20 were tested for regulatory activity with a *Renilla* luciferase assay. For a 711 base pair noncoding fragment encompassing the most strongly associated variant, rs1884302, the luciferase assay showed that the risk allele (C) of rs1884302 drives higher expression of the reporter than the common allele (T). When this same DNA fragment was tested in zebrafish transgenesis studies, a strikingly different expression pattern of the green fluorescent reporter was observed depending on whether the transgenic fish had the risk (C) or the common (T) allele at rs1884302. The in vitro results suggest that altered *BMP2* regulatory function at rs1884302 may contribute to the etiology of sagittal nonsyndromic craniosynostosis. The in vivo results indicate that differences in regulatory activity depend on the presence of a C or T allele at rs1884302.

KEYWORDS
BBS9, BMP signaling, BMP2, craniofacial development, craniosynostosis, enhancer

INTRODUCTION

Craniosynostosis (CS) is a common congenital anomaly caused by the premature closure of one or more of the cranial vault sutures (Cohen, 2000; Johnson & Wilkie, 2011). Mutations in at least twelve genes are found in syndromic craniosynostosis: *EFNB1*, *ERF*, *FGFR1*, *FGFR2*, *FGFR3*, *MSX2*, *POR*, *PTH2R*, *RAB23*, *SIX2*, *TCF12*, and *TWIST1* (Lattanzi, Barba, Di Pietro, & Boyadjiev, 2017; Passos-Bueno, Serti Eacute, Jehee, Fanganiello, & Yeh, 2008). Approximately, 80% of cases with craniosynostosis do not present with other birth defects or developmental delay and their etiology remains largely unknown.

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The most common form of nonsyndromic craniosynostosis (NCS) involves the sagittal suture and comprises 40–58% of all NCS cases (Boyadjiev, 2007; Kimonis, Gold, Hoffman, Panchal, & Boyadjiev, 2007) with a prevalence of 1.9–2.3 per 10,000 live births (Hunter & Rudd, 1976; Lajeunie, Le Merrer, Bonaiti-Pellie, Marchac, & Renier, 1996). In our previous genome-wide association study (GWAS) of sagittal NCS (sNCS), significant associations were observed in a 120 kilobase (kb) region located 345 kb downstream of *BMP2* on chromosome 20p.12.3, spanning rs1884302 ($p = 1.13 \times 10^{-14}$) to rs6140226 ($p = 3.4 \times 10^{-11}$) and in a 167 kb intronic region of *BBS9* on chromosome 7p14.3 between rs10262453 ($p = 1.61 \times 10^{-10}$) and rs17724206 ($p = 1.50 \times 10^{-8}$). The associations for rs1884302 and rs10262453 were replicated in an independent population of 172 unrelated sNCS probands and 548 unaffected controls ($p = 4.39 \times 10^{-31}$ and $p = 3.50 \times 10^{-14}$, respectively) (Justice et al., 2012).

It is known that the formation and maintenance of the cranial sutures depends on osteoblast and osteocyte activity, which are controlled by several regulatory factors, including the bone morphogenetic proteins (BMPs). Recent animal studies have demonstrated that high levels of BMP2 in rabbits (Kinsella et al., 2011) and enhanced BMP2 signaling driven by *BMPR1A* in neural crest cells in mice (Komatsu et al., 2012) result in premature suture fusion.

Because no deleterious mutations were identified in the coding regions of *BMP2* in the current study, we hypothesized that BMP2 expression is abnormally regulated in sNCS patients. To test this hypothesis, two conserved noncoding regions near *BMP2* were tested for regulatory activity using a *Renilla* luciferase assay. Zebrafish transgenesis studies were then performed with the common (T) and risk (C) alleles at rs1884302 in order to investigate the regulatory activity of this noncoding variant.

MATERIALS AND METHODS

Luciferase assay

Two putative regulatory regions, R1 and R2, were identified and amplified from a sNCS patient (11028–1). R1 was 1,287 base pairs (bp) in length and located 357 kb downstream from *BMP2* (GRCh37/hg19, chr20: 7,117,908–7,119,194, with 100% identity between fragment and reference sequence); R2 was 711 bp in length and located 344 kb downstream of *BMP2* (GRCH37/hg19, chr20: 7,105,873–7,106,588, with 99.5% identity between fragment and reference sequence) and encompassed rs1884302. Patient (11028–1) was heterozygous (C/T) at rs1884302, and the R2 C allele and R2 T allele fragments were identical in sequence except at rs1884302. The SacI restriction enzyme site was introduced at the 5′ end and the MluI restriction enzyme site was introduced at the 3′ end to the fragments. R1 primer set: 5′-TTGAGCTCGAGCACATACACAACATGGC-3′ and 5′-TTACGCGTGTGTCTGTCATTTCTTTCTGTCCTG-3′.R2 primer set: 5′-TTGAGCTCGTTCTAGATCCCTGAGGAGTCG-3′ and 5′-TTACGCGTCAAGATGGCGAACTCACATC-3′. PCR amplified fragments were digested with SacI and MluI and ligated into sites upstream of a *BMP2* promoter construct (product ID: S710234, switchgeargenomics) with a *Renilla* luciferase reporter gene. The pGL4.17 [luc2/Neo] vector that can express firefly luciferase (Promega) was used as a transfection control. The *BMP2* promoter–*Renilla* luciferase construct was co-transfected with pGL4.17 [luc2/Neo] to a human osteosarcoma cell line (MG-63, ATCC) using Lipofectamine reagent (Invitrogen). Luminescence was produced by the Dual-Luciferase Reporter Assay System and measured with a Turner BioSystems™ 20/20 Luminometer. Luminescence of *Renilla* luciferase was normalized with that of firefly luciferase. Untransfected cells produced negligible levels of luminescence. Three independent transfections were performed. Each transfection set included the empty vector, R1, R2(T), and R2(C) and was performed independently 11 times.

Zebrafish transgenesis

The R2 fragments containing the common and risk variants of rs1884302 were cloned in the zebrafish enhancer detection vector (ZED vector) (Bessa et al., 2009). The ZED vector is a Tol2 transposon based vector with insulator sequences that minimize positional effects; it contains a cardiac actin promoter, which drives red fluorescent protein (RFP) allowing for identification of transgenic embryos, and green fluorescent protein (GFP), which functions as a reporter for regulatory activity of the candidate enhancer (Bessa et al., 2009). Tol2 mRNA was synthesized with the mMessage mMachine SP6 kit (Ambion) using NotI linearized pCS2FA-transposase vector as template DNA (Kwan et al., 2007). Microinjections of both constructs were performed individually into 1-cell stage zebrafish embryos using 20 picograms (pg) of plasmid DNA mixed with 50 pg of Tol2 mRNA. Embryos were incubated at 28°C with 0.003% PTU (1-phenyl 2-thiourea) to suppress pigmentation. Transgene integration was identified by RFP expression in the skeletal muscle at 48 hr post fertilization (hpf) and somatic GFP expression was observed from 48 hpf to 72 hpf. RFP positive embryos from both constructs were grown to adulthood and screened for germline transmission of the transgene. Two to three germline transmitting founders for each construct were identified and progeny from stable F1 Lines were screened for GFP expression from 48 hpf to 72 hpf. The C and T alleles of the transgene were validated by PCR and sequencing of the RFP/GFP positive embryos from each germline transmitting founder. The only sequence difference was at the rs1884302 locus. Screening and imaging of embryos was performed using either inverted or confocal microscopy.

RESULTS

Luciferase assay

Two conserved regions (Supplementary Figure S1) identified in the associated region on chromosome 20 (Justice et al., 2012) were cloned 5′ upstream of a *BMP2* promoter reporter construct (Figure 1a). The first region tested (R1) was 1,287 bp long and in a DNase hypersensitive region with several potential transcription factor binding sites. The second region tested (R2), a 711 bp long fragment,

encompassed rs1884302, the SNP with the strongest association to sNCS (Justice et al., 2012). An increase in luciferase reporter was observed for R1 ($p = 0.03$), relative to the empty control, although the difference was not significant at a nominal critical value of 0.01 (Figure 1b). Both R2 fragments, with either the risk allele (C) or the common allele (T) at rs1884302, had higher expression levels of the luciferase reporter relative to the control; and the risk allele (C), which was over-transmitted in our sNCS cohort, had higher expression than that of the common allele (T). The expression of the luciferase reporter for the R2 fragment with the C allele was highly significant ($p = 0.0016$) whereas the R2 T allele fragment was only marginally significant ($p = 0.045$) at a nominal critical level of 0.05. However, the two allele fragments did not differ significantly in their modulation of the promoter activity ($p = 0.16$), most likely due to the relatively small sample size, although the trend for the expression levels was consistent with the risk allele (C) having the highest expression. After adjustment for multiple tests, only the risk allele (C) compared to the control remained significant at a critical value of 0.01.

Zebrafish transgenesis

A zebrafish transgenesis assay was performed to test the 711 bp R2 fragment for putative regulatory function in vivo using the ZED vector (Bessa et al., 2009), which contains insulators that minimize false positive expression due to position effects. The risk allele (C) and common allele (T) fragments were tested, and pronounced GFP expression in the head region for the transgenic fish with the C allele was observed, indicating the 711 bp fragment had regulatory function. Because of the mosaic expression of GFP in the injected fish, stable lines were generated for both constructs by screening founders for germline transmission. These F1 adults were outcrossed and GFP expression was observed in the F2 progeny during embryogenesis. Progeny were evaluated from five founders: two founders for the C allele construct (C1 and C2) and three founders for the T allele construct (T1, T2, and T3).

There was clear GFP expression in the C allele fish (progeny of C1 and C2; Figure 2a), and no GFP in the progeny from two of the T allele fish founders (T1 and T2; Figure 2b). There was some GFP expression in the progeny of the T3 fish (T allele), but the expression pattern was clearly different than that observed for the C allele fish (Figure 2). GFP expression in the C-allele fish occurred in the midbrain and hindbrain, while in the T allele fish from founder T3, GFP expression occurred in the midbrain-hindbrain boundary. Overall, the zebrafish transgenesis data suggests a regulatory role for the 711 bp fragment that is affected by the change from the T to the C allele at rs1884302.

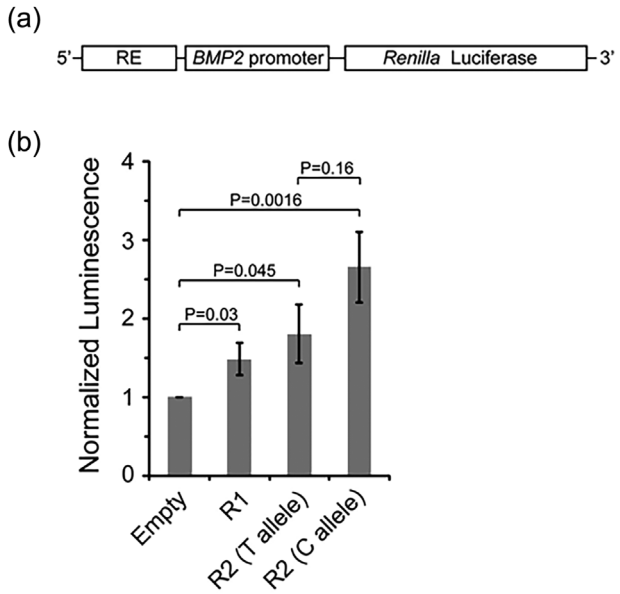

FIGURE. 1 *Renilla* luciferase assay. (a) RE denotes a putative regulatory element inserted at this position. Fragments R1 (GRCh37/hg19, chr20: 7,117,908–7,119,194) and R2 (GRCH37/hg19, chr20: 7,105,873–7,106,588) were cloned into the RE region. (b) Luciferase reporter assay with and without a putative regulatory region. Bars represent mean ± SE

DISCUSSION

Our results are consistent with the idea that regions near the *BMP2* locus identified by our GWAS contain a regulatory element(s) for *BMP2* expression. The luciferase assay results suggest that the rs1884302 SNP may itself be an enhancer that exerts a direct regulatory effect on *BMP2*. Although the only difference in sequence between the two R2 fragments tested in our luciferase assay was at rs1884302, other SNPs or copy number variants near rs1884302 may impact the expression of *BMP2* independently from or in concert with this SNP.

The zebrafish transgenesis results suggest a role of the C allele at rs1884302 in the regulatory function of the 711 bp noncoding DNA fragment. This assay tests a potential regulatory function of defined DNA segments in an in vivo vertebrate model, however, due to its limitations, we were unable to make any conclusions on the regulation of endogenous *BMP2* expression or its effect on osteogenesis of the skull vault that occurs at 27 days past fertilization (Quarto & Longaker, 2005). Again, the only difference in sequence between the fragments used in transgenesis was at rs1884302. Although there appears to be no sequence conservation in this 711 bp fragment between humans and zebrafish, there is a 21 bp region which aligns perfectly in zebrafish, and is intronic to *bmpr1ba* (Supplementary Figure S2).

Transcription factor binding sites (TFBS) created by a change to a C allele at rs1884392 were identified using MEME (Bailey et al., 2009) and TOMTOM (Gupta, Stamatoyannopoulos, Bailey, & Noble, 2007). One TFBS candidate binds *RFX7*, which plays an important role in the molecular cascade that controls ciliogenesis in the neural tube (Manojlovic, Earwood, Kato, Stefanovic, & Kato, 2014). This is of interest because we identified a strong association in our sNCS GWAS to *BBS9* (Justice et al., 2012), a gene involved in the ciliopathies (Veleri

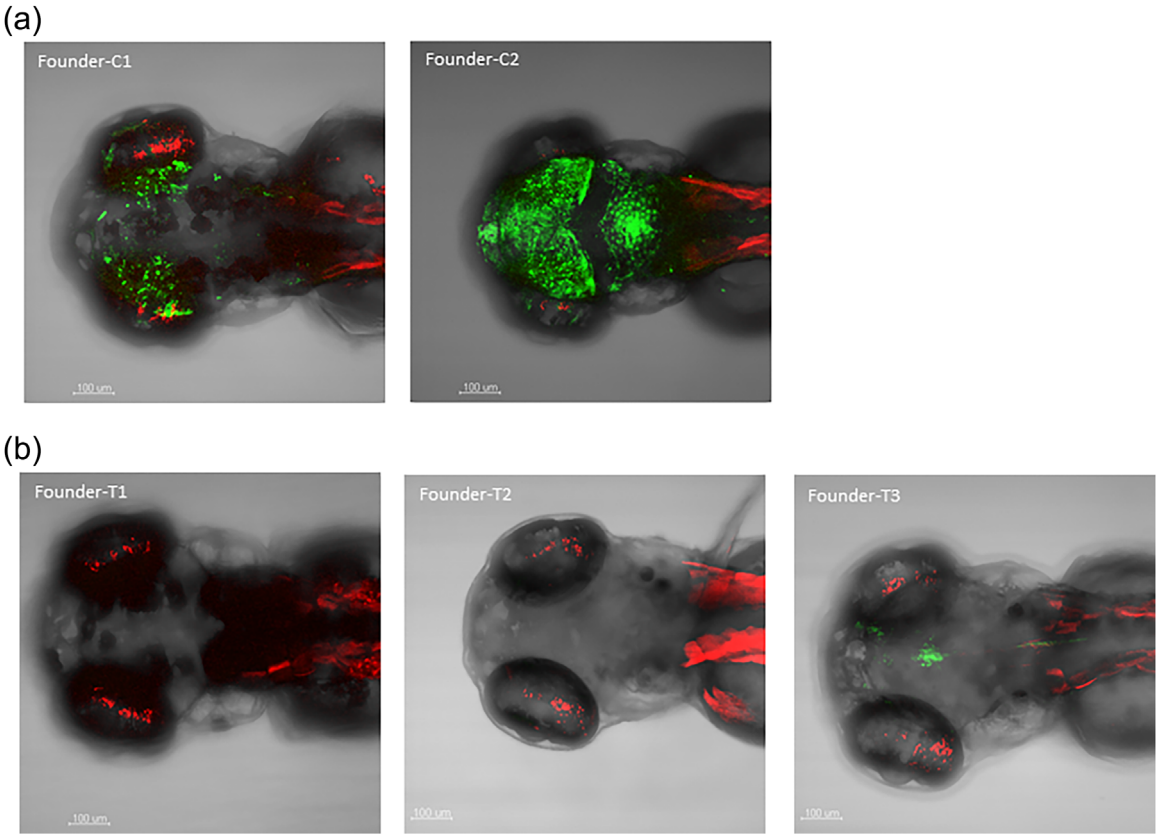

FIGURE. 2 Comparison of GFP expression in transgenic zebrafish embryos with C vs. T allele. Representative images showing dorsal views of 72 hpf embryos from each of the five founders. RFP expression indicates successful transgene integration (a) Embryos from progeny of two risk allele (C) founders. (b) Embryos from progeny of three common allele (T) founders. All embryos are oriented with their heads to the left side.

et al., 2012). Another TFBS candidate binds *SMAD3* and the finding that *BMP2* stimulates the SMAD2/3 pathway (Wang et al., 2014) is consistent with the idea that increased BMP2 production is due to SMAD-signaling.

Our results suggest that over-activation of BMP2 signaling contributes to the molecular basis of sNCS. The noncoding SNP rs1884302 appears to act as an enhancer involved in regulation, with clear differences in expression dependent on the presence of the risk or common allele. These findings, as well as the reports of *Gdp6* (Settle et al., 2003), *Bmp4* (Cooper et al., 2010), *Bmp3* (Schoenebeck et al., 2012), and *Bmpr1a* (Komatsu et al., 2012) all involving craniosynostosis in animal models, suggest that the BMP signaling pathway plays a role in craniosynostosis. A recent study (Whitton et al., 2016) reported *BMP2* RNA overexpression in five of six osteoblasts isolated from the fused sutures of sNCS cases, further indicating that *BMP2* over-expression may be responsible for sNCS.

Due to high frequency of this allele in the population (0.328 in Europeans, www.1000genomes.org), it seems likely that the C allele at rs1884302 alone is not responsible for the phenotype, but that an interaction of this allele with other variant(s) may be responsible for the sNCS phenotype. A recent report found that seven percent of probands with midline NCS (sagittal and metopic NCS) had damaging mutations in *SMAD6* with strikingly reduced penetrance, and that 14 out of 17 affected individuals also carried the C risk allele at rs1884302. None of the 10 parents with *SMAD6* mutations and a T allele at rs1884302 showed evidence of craniosynostosis (Timberlake et al., 2016). Because the frequency of the C risk allele is high, the finding of 14 affected carriers with a C risk allele could be due to chance. However, it does suggest a two-locus inheritance for some sNCS cases, which may require characterizing the regulatory region surrounding rs1884302 and screening probands for mutations in genes involved in the *BMP2* signaling pathway.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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