Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Human-specific changes in two functional enhancers of *FOXP2*

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ARTICLE INFO

Short Communication

Article history:
Received: September 17, 2022
Accepted: November 20, 2022
Published: November 30, 2022

Keywords:

Human evolution; language evolution; FOXP2; enhancers; Neanderthals; SMARCC1

ABSTRACT

FOXP2 is a gene involved in language development and function. Neanderthals and humans share the same coding region of the gene, although the formers are thought to have exhibited less sophisticated language abilities. In this paper, we report on several human-specific changes in two functional enhancers of *FOXP2*. Two of these variants are located within the binding sites for the transcription factors POLR2A and SMARCC1, respectively. Interestingly, SMARCC1 is involved in brain development and vitamin D metabolism. We hypothesize that the human specific change in this position might have resulted in a different regulation pattern of *FOXP2* expression in our species compared to extinct hominins, with a potential impact on our language abilities.

Doi: http://dx.doi.org/10.14715/cmb/2022.68.11.3

Introduction

Mutations in the coding region of *FOXP2* are known to cause speech and language problems, including diverse forms of apraxia and dyspraxia (1-3). Recent in silico analyses have further uncovered several missense single nucleotide polymorphism (SNPs) with a detrimental effect of the structure and function of the FOXP2 protein, and potentially associated to diverse clinical conditions (4). *FOXP2* contributes to a sensorimotor loop important for auditory-vocal control, as well to a motor-skill learning loop important for speech production, via its effects on neural plasticity (5). Although extinct hominins (specifically, Neanderthals and Denisovans) are thought to have exhibited less advanced language abilities compared to humans, the FOXP2 protein is identical in the three species (6,7). In birds, differential regulation of the gene is known to impact on song performance (8) and song learning abilities (9). Accordingly, differences in the expression pattern of *FOXP2* between extinct hominins and modern humans could be hypothesized to account for some of the aforementioned differences in their language abilities. Interestingly, Neanderthals bear the ancestral allele of a binding site for the transcription factor POU3F2, which results in increased expression of *FOXP2* (10). Duplications of the *FOXP2* region, also resulting in higher levels of FOXP2, are known to give rise to autistic features in patients, as well as delayed speech and language development (e.g. DECIPHER patient 804). As noted, less sophisticated language abilities, but also autistic-like features (i.e. a less flexible cognition) have been hypothesized for Neanderthals (11-15).

We have recently identified two functional enhancers of *FOXP2* in the intergenic region between *FOXP2* and the adjacent *MDFIC* gene: FOXP2-E^{proximal} (chr7: 114,456,873-114,463,136, hg19) and FOXP2-E^{distal} (chr7:114,541,370-114,543,683, hg19) (16). Deletion of either of these two enhancers in the SK-N-MC neuroblastoma cell line downregulates *FOXP2* and reduces the amount of FOXP2 protein. This suggests that both enhancers might upregulate *FOXP2* in brain cells. In this paper, we report on several human-specific SNPs in these two enhancers compared to extinct hominins. We hypothesize about the impact of these differences on *FOXP2* expression and ultimately, on the functions to which this gene contributes.

Materials and Methods

The identification, characterization, and functional validation of the two *FOXP2* enhancers were performed as described in (16). We used the UCSC/Penn State Bioinformatics comparative genomics alignment pipeline for aligning the vertebrate sequences. Additionally, we used the Vertebrate Multiz Alignment & Conservation track of the UCSC Genome Browser for estimating the degree of conservation of the enhancers (only the species displayed

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in Figure 1 were considered). Because there are no reference genomes for extinct hominins in the same way that there is a modern human reference genome, we downloaded the variant files (VCF) of the Altai Neanderthal, the Vindija Neanderthal, and the Denisovan individual from the Neanderthal project website (http://cdna.eva.mpg.de/neandertal/Vindija/VCF). We then used FastaAlternateReferenceMaker of GATK 3.7 (17) to obtain a consensus sequence of the three extinct hominin genomes using the GRCh37 as reference. The resulting sequences were aligned with Clustal Omega (18), with the aim of identifying variant positions. We used Gimp (version 2.8.22-1) and creative-commons materials for generating the graphics accompanying the alignments in Figure 1. The regulatory properties of the enhancers (including the binding sites for transcription factors) were determined with the Encyclopedia of DNA Elements (ENCODE, https://genome.ucsc.edu/ENCODE/).

Results

In order to know more about the degree of conservation of the two *FOXP2* enhancers, we first compared their sequence in vertebrate species known to show some form of imitative vocalization, even if rudimentary, given the role played by *FOXP2* in vocal learning. This included 3 songbirds (zebra finch, collared flycatcher, and white-throated sparrow), 2 cetaceans (dolphin and killer whale), 5 bats (black flying-fox, megabat, David's myotis, microbat, and big brown bat), 4 extant primates (chimp, gorilla, hamadryas baboon, and human), 1 rodent (mouse), and 2 extinct hominins (Neanderthal and Denisovan). We used the horse, the chicken, the Carolina anole, and the Western clawed frog as controls. We then looked for sequence differences between archaic and modern humans, with a focus on differences predicted to affect the binding site of transcription factors of *FOXP2*.

We found that although these two enhancers are absent in songbirds, FOXP2-E^{proximal} is more conserved than FOXP2-E^{distal}. Accordingly, FOXP2-E^{proximal} is conserved in all primates and partially conserved in bats, cetaceans, and the mouse, whereas FOXP2-E^{distal} is only partially conserved in the baboon (Figure 1). Regarding extinct hominins, we found several differences between the human reference genome (GRCh37) and the Altai Neanderthal, the Vindija Neanderthal, and the Denisova genomes. The Denisovan FOXP2-E^{proximal} exhibits 5 fixed or nearly fixed (frequency > 0.99 in present-day human populations) differences with the modern human sequence (two ancestral and three derived [red bars in Figure 1]), with one of these differences (114460158) being located within the predicted binding site for the transcription factor SMARCC1. In both Neanderthal genomes, FOXP2-E^{proximal} shows four fixed or nearly fixed differences with the modern human enhancer (two ancestral and two derived [red bars in Figure 1]), with two of them located within the predicted binding sites for the transcription factors POLR2A (114459001) and SMARCC1 (114460158). Three differences are shared by the three extinct hominin genomes: in positions 114460158 and 114462035 Neanderthals and Denisovans exhibit the ancestral alleles, whereas in position 114461593 they exhibit a derived allele (A>T). Accordingly, the most interesting difference between extinct hominins and modern humans concerns to position 114460158, where modern humans exhibit a derived T>G. As noted, this position is located within the binding site for SMARCC1. Regarding FOXP2-E^{distal}, we found no differences between the Denisovan sequence and the modern human sequence. In the case of Neanderthals, we found two fixed differences, although they lay outside the predicted binding sites for known transcription factors (Figure 1).

Discussion

FOXP2 is a gene important for speech and language development and evolution (19,20). The FOXP2 protein is identical in modern humans, Neanderthals, and Denisovans. Nonetheless, we have identified a human-specific change in the binding site for SMARCC1 within one functional enhancer of the gene (FOXP2-E^{proximal}). SMARCC1 is a component of the large ATP-dependent chromatin remodelling complex SNF/SWI and plays an important role in the development of the forebrain, particularly, in neurogenesis (21). This regulatory mechanism also involves *PAX6*, which controls *FOXP2* expression (22). *PAX6* has been hypothesized to account for some of the cognitive differences between Neanderthals and humans (23). SMARCC1 also mediates the effects of vitamin D on gene transcription via its association with the WINAC complex (a chromatin-remodeling complex recruited by VDR, the vitamin D receptor) (24). Interestingly, in mice, vitamin D deficiency reduces the amount of Foxp2-expressing cells in the developing cortex (25). Interestingly too, core candidates for the evolution of language have been related to vitamin D homeostasis and function (23). Western European Neanderthals have been claimed to suffer from vitamin D deficiency (26). In present-day human populations, low vitamin D levels are found in people with conditions impacting on language abilities, specifically autism (27). Overall, this evidence points to some recent change in a regulatory mechanism of FOXP2 expression mediated by the chromatin-remodelling factor SMARCC1, involving vitamin D homeostasis, and impacting on brain development and cognition, including language abilities. We expect that our *in silico* analysis helps to achieve a better understanding of the evolutionary modifications in the regulatory landscape of *FOXP2* with an impact on speech and language evolution. Still, functional studies are needed to confirm the hypothesis outlined above. One promising approach would be mimicking the Neanderthal/Denisovan difference in the binding site for SMARCC1 in the same human neuroblastoma cell line that we have previously for testing the functionality of these two human enhancers, with the aim of checking its effects *in vivo* and knowing more about its biological significance.

Conflicts of Interest declaration

The authors declare that they have no conflict of interest.

Author Contributions

ABB conceived and wrote the paper. RTR and SRP performed the molecular analyses. PG and CLF retrieved the extinct hominin data and performed the comparative analyses of the DNA sequences. PGB contributed to the discussion of the findings.

Data availability Statement

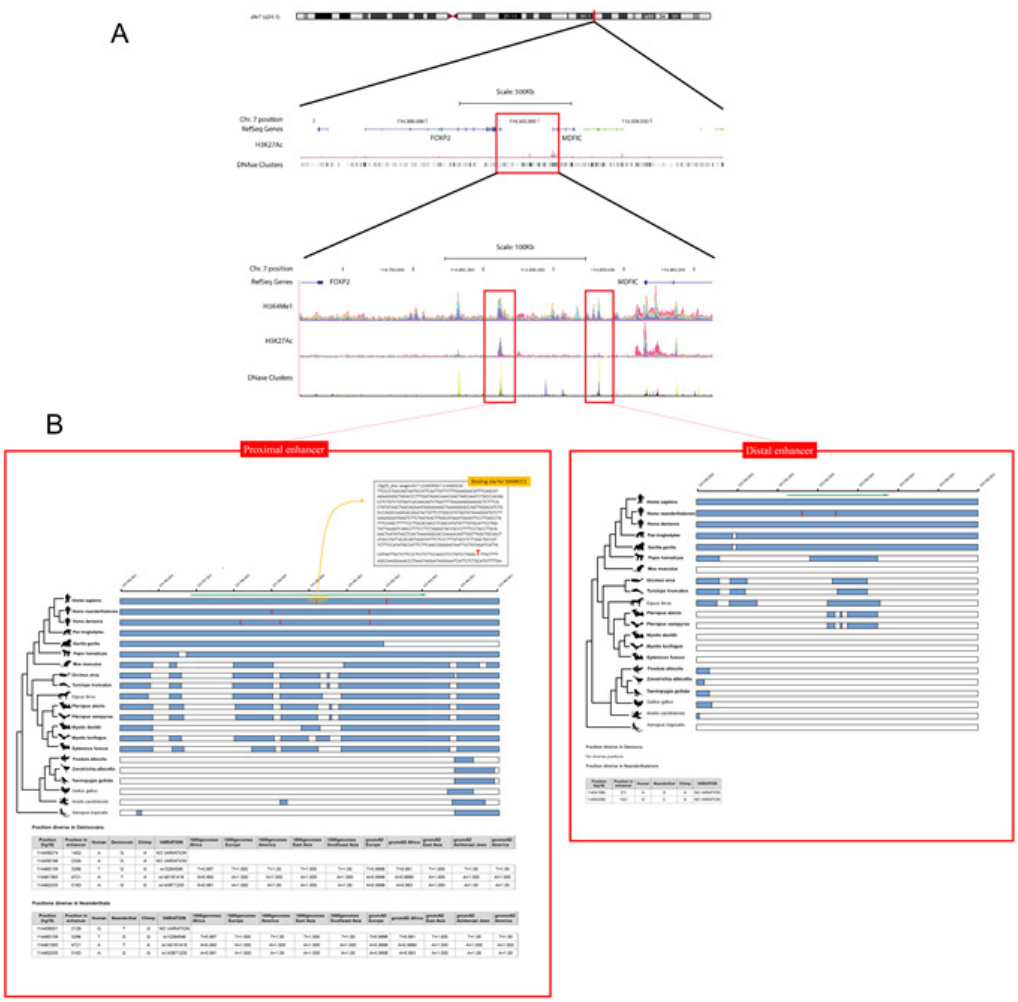

Figure 1. Changes with a potential evolutionary significance in two enhancers of *FOXP2*. A. Snapshot of an ENCODE UCSC genome-browser search showing the genomic location of the two enhancers (red boxes), which are placed downstream *FOXP2* and upstream *MDFIC*. The figure also shows three common hallmarks of cis-regulatory elements of gene expression: H3K4Me1 marks, H3K27Ac marks, and DNAse clusters (reproduced from (16)). B. Sequence comparison of the two enhancers in selected vertebrate species showing evidence of vocal imitation and/or learning. The green arrow shows the position of the enhancers within chromosome 7. The figure includes 2 kb of the flanking sequences, to show the degree of conservation of this region. The trees on the left of each alignment represent the phylogenetic relationships between the species. Species for which there is evidence of imitative vocalization, even if rudimentary, are in bold. The blue fractions in the alignments represent the regions that are homologous to the human sequence, whereas the red lines represent the positions that are derived in modern humans or extinct hominins compared to chimps. The tables at the bottom report on the positions that show differences in modern humans vs. extinct hominins, including their frequencies in present-day human populations. For *SMARCC1*, we provide the full binding sequence within the FOXP2-E-^{proximal} enhancer. The derived T found in modern humans is highlighted in red.

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Compliance with Ethical Standards

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Funding Sources

This research was supported by grant PID2020-114516GB-I00 funded by MCIN/AEI/ 10.13039/501100011033 (to ABB).

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