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ScienceDirect
Molecular Genetics and Metabolism 90 (2007) 97–111
Molecular Genetics and Metabolism
www.elsevier.com/locate/ymgme

Functional analysis of mutations in *TGIF* associated with holoprosencephaly

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Received 26 July 2006; accepted 26 July 2006
Available online 7 September 2006

Abstract

Holoprosencephaly (HPE) is the most common structural malformation of the forebrain and face in humans. Our current understanding of the pathogenesis of HPE attempts to integrate genetic susceptibility, evidenced by mutations in the known HPE genes, with the epigenetic influence of environmental factors. Mutations or deletions of the human *TGIF* gene have been associated with HPE in multiple population cohorts. Here we examine the functional effects of all previously reported mutations, and describe four additional variants. Of the eleven sequence variations in TGIF, all but four can be demonstrated to be functionally abnormal. In contrast, no potentially pathogenic sequence alterations were detected in the related gene *TGIF2*. These results provide further evidence of a role for TGIF in HPE and demonstrate the importance of functional analysis of putative disease-associated alleles.
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Keywords: TGIF; TGIF2; Corepressor; HPE; Complex inheritance

Introduction

Holoprosencephaly (HPE) is best understood as a failure in the generation of, or response to, midline signals that normally instruct the developing prosencephalon to divide into paired left and right hemispheres and subcortical structures [1,2]. This incompletely understood process is etiologically heterogeneous and can be perturbed by both genetic and environmental causes, either individually or more likely in combination. Clinically, there is a nearly continuous spectrum of malformations consistent with HPE, and this variable expressivity and/or penetrance is clearly demonstrable in all well documented familial cases segregating a particular HPE mutation. Families frequently manifest a wide range of phenotypes, such as, typically severe HPE with perinatal lethality, or microforms (such as microcephaly, closely spaced eyes, single central incisor), or even clinically unaffected individuals. Most investigators consider HPE to be consistent with autosomal dominant inheritance of a major susceptibility locus, although X-linked, autosomal recessive, and digenic inheritance have been suggested in isolated cases [3,4]. With few exceptions, such as ZIC2 and GLI2 [5–7], prediction of phenotype based on the type or nature of mutation has been elusive for HPE, as well as for an increasingly large number of unrelated genetic disorders [8,9]. We hypothesized that for some conditions, including HPE, alterations in modifier genes or interactions with environmental factors contribute

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1096-7192/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.ymgme.2006.07.011

to the variable phenotype in HPE and other disorders (multiple–hit hypothesis) [10]. *TGIF* [11] is one of several genes associated with HPE, including *Sonic Hedgehog* (*SHH*), *SIX3*, *ZIC2*, *GLI2* and potentially *TDGF1* and *PATCHED* [5–7,12–16]. Although several of these genes are within the Sonic Hedgehog pathway, having been studied because *SHH* was the first HPE gene to be identified, there is currently limited understanding of the potential interactions between these HPE susceptibility genes. In all cases to date, only heterozygous sequence changes or hemizygosity have been detected in these genes.

TGIF (5′TG3′) interacting factor (or TGFβ-induced factor, OMIM #602630) is a transcriptional repressor and member of the TALE (Three aminoacid loop extension) class of atypical homeodomain proteins [17]. The *TGIF* gene is located on 18p11.3 within the *HPE4* minimal critical region (OMIM #142946) defined by the comparison of several cytogenetic rearrangements leading to the loss of 18p in association with the presence of HPE or its microforms [18,19]. Mutations in the human *TGIF* gene have been identified exclusively among HPE patients in several studies and include deletions of the entire gene due to cytogenetically visible [20] or microscopic rearrangements [21,22], missense and nonsense sequence changes [11,23,24]. However, attempts to model HPE in mice utilizing targeted inactivation of the *Tgif* gene have failed to recapitulate the clinical findings of HPE seen in humans [25,26,52,53, and this study]. Consequently, it became important for us to determine the potential mutational spectrum of the *TGIF* and the related *TGIF2* genes [27–29] among our HPE patients. Furthermore, it is important to evaluate the functional effects of these putative disease-associated alleles, since these findings have a direct bearing on genetic counseling, as well as evaluations aimed at the study of gene–gene and gene-environment interactions.

TGIF was first identified as a 272 aminoacid (NP_775300) homeodomain transcription factor that competed with the binding of RXR to the DR-1 RXRE in the rat cellular retinol binding protein II (CrbpII) promotor [30,31]; however, the physiological significance of this competition is poorly understood, since most retinoid response elements lack direct binding sites for TGIF. Subsequent investigations have described multiple modes of repression for TGIF, affecting the magnitude of TGFβ-induced responses as a corepressor of Smad2 or Smad3 [32–35], as well as a distinct role as a corepressor of retinoic acid mediated changes in gene expression through interactions with RXR nuclear receptors [26]. Recent studies demonstrate that TGIF also has a more general role as a corepressor of RXR nuclear receptors through a protein–protein interaction, and the recruitment of additional corepressors into a multiprotein complex. Similar multiprotein complexes are seen with Smad2 and TGIF, and can include the recruitment of histone deacetylase (HDAC), CtBP and mSin3. These studies suggest that the repression effects are likely to be mediated by gene-targeted changes in chromatin remodeling. In general, TGIF acts to attenuate, or limit the extent of TGFβ or retinoid responses; however, limited information is available on a potential direct role as a transcription factor, beyond its ability to participate as a molecular switch from gene activation to gene repression. There may well be other functions of TGIF and TGIF2 yet to be described.

Members of the TGFβ family of secreted signaling molecules play diverse roles in intercellular signaling and developmental programs (reviewed in [36]). These TGFβ ligands bind to heterodimeric Type I/Type II cell surface receptors leading to the phosphorylation, and activation of Smad 2 and/or 3. These activated Smads bind to the common Smad 4 and translocate to the nucleus to influence gene expression. Activated Smad complexes are directed to transcriptional targets through interactions with additional transcription factors, such as FoxH1, and via direct DNA binding by the Smads. Target genes can be activated in part by the recruitment of coactivators, such as p300/CBP, and the extent of this activation can be limited by interactions with corepressors like TGIF. It is the balance between competing activator and repressor activities that ultimately determines the magnitude of the TGFβ response within the cell.

It was initially attractive to speculate that mutations in TGIF affected the functioning of Nodal [11], or related TGFβ factors, since genes in these pathways are intimately involved in the development of the vertebrate organizer and its midline derivatives (such as the notochord and prechordal plate) that are considered essential organizing centers for specification of all three vertebrate axes. Defective Nodal signaling can result in cyclopic phenotypes that can resemble some of the more extreme forms of HPE seen in humans. Given the role of TGIF as a corepressor, loss of TGIF function would lead to an increase, rather than a decrease in signaling. However, it may be that too much or too little Nodal signaling could result in defects in axis formation and potentially generate HPE-like phenotypes.

The second described role for TGIF, as a modulator of retinoid responses, is perhaps a more attractive model since retinoic acid is a well-described teratogen resulting in HPE both in humans and animal models. Furthermore, targeted disruption of murine *Tgif* leads to aberrant activation of retinoid-responsive gene expression and an increase in sensitivity to excess retinoids [26]. However, even complete elimination of Tgif function in homozygous null mice does not fully reflect the morphological abnormalities attributed to the loss of a single *TGIF* allele in humans [25,26,52,53]. The potential basis of these species differences (mouse vs. human) is poorly understood. Interestingly, there are also minor phenotypic differences between mouse lines established in different laboratories, which are attributed to genetic modifiers between strains.

We set out to examine the mutational spectrum of the human *TGIF* gene and putative promoter region using a more highly sensitive method of dHPLC screening than had been used in our original studies; we applied the same technology to the evaluation of *TGIF2* as a potential candidate