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NOTCH1 missense alleles associated with left ventricular outflow tract defects exhibit impaired receptor processing and defective EMT

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ABSTRACT

Notch signaling is essential for proper cardiac development. We recently identified missense variants in the NOTCH1 receptor in patients with diverse left ventricular outflow tract (LVOT) malformations (NOTCH1^{G661S} and NOTCH1^{A683T}) that reduce ligand-induced Notch signaling. Here, we examine the molecular mechanisms that contribute to reduced signaling and perturbed development. We find that NOTCH1^{A683T} exhibits reduced S1 cleavage due to impaired trafficking through the endoplasmic reticulum (ER). This observation is consistent with improper localization of the variant receptor to the ER and decreased presentation at the cell surface. In contrast, the nearby mutation NOTCH1^{G661S} exhibits reduced cell-surface presentation in the absence of overt folding or trafficking defects. To examine the implications of these variants in disease pathogenesis, we investigated their effect on epithelial-to-mesenchymal transition (EMT), a critical process for development of the outflow tract. We find that these LVOT-associated *NOTCH1* alleles can contribute to defective EMT in endothelial cell lines through impaired induction of Snail and Hes family members. These data represent the first description of a molecular mechanism underlying *NOTCH1* mutations in individuals with LVOT malformations, and have important implications regarding the functional contribution of these alleles to a complex set of developmental defects.

1. Introduction

Defects involving the left ventricular outflow tract (LVOT) comprise a clinically significant group of congenital cardiovascular malformations. LVOT malformations, including bicuspid aortic valve (BAV), aortic valve stenosis (AVS), coarctation of the aorta (COA), and hypoplastic left heart syndrome (HLHS), are present in 1 in 1000 live births, and account for a significant portion of infant mortality [1–3]. Although the etiology of LVOT malformations is unclear, both environmental and genetic components play a role in disease pathogenesis. For example, prenatal exposure to solvents or high phenylalanine levels (secondary to maternal phenylketonuria) have been associated with higher incidence of LVOT malformations [4]. In addition, linkage analysis demonstrates a strong genetic component for these malformations. A non-parametric linkage analysis of LVOT malformation shows linkage to three chromosomes with overlapping linkage peaks suggesting a common genetic cause [5]. Mutations in *NOTCH1* have been reported in two families with bicuspid aortic valve (BAV) and calcific AVS [6], in sporadic BAV [7,8], and by our group in BAV, AVS, COA, and HLHS [9]. In support of the presumed common genetic pathogenic mechanism, we identified *NOTCH1* missense variants in patients across the LVOT phenotypic spectrum and found that these alleles reduce ligand-dependent Notch signaling [9].

Notch signaling is an evolutionarily conserved pathway that regulates cell fates and tissue formation during embryogenesis, including cardiac development [10,11]. The NOTCH1 receptor is synthesized as a large polypeptide with 36 EGF-like repeats in the extracellular domain, three NOTCH/Lin repeats, a transmembrane domain, a transactivating domain, and intracellular domain with six ankyrin repeats to facilitate protein–protein interactions. Mammalian NOTCH1 is synthesized as a single 300-kDa polypeptide in the endoplasmic reticulum (ER) and cleaved by a furin convertase during posttranslational processing in the Golgi complex into p120 and p180 (S1 cleavage, see Fig. 1A). Following cleavage, the two portions of the protein are presented as a functional heterodimer on the cell surface. Ligands of the Delta and Jagged families presented on adjacent cells can interact with the extracellular domain of NOTCH1. This interaction triggers two subsequent cleavages (S2 and S3), resulting in the release of the intracellular domain (NICD). NICD translocates into the nucleus, where it functions in the activation of downstream targets including members of the Hairy-Enhancer of Split (Hes) family of transcription factors [12].

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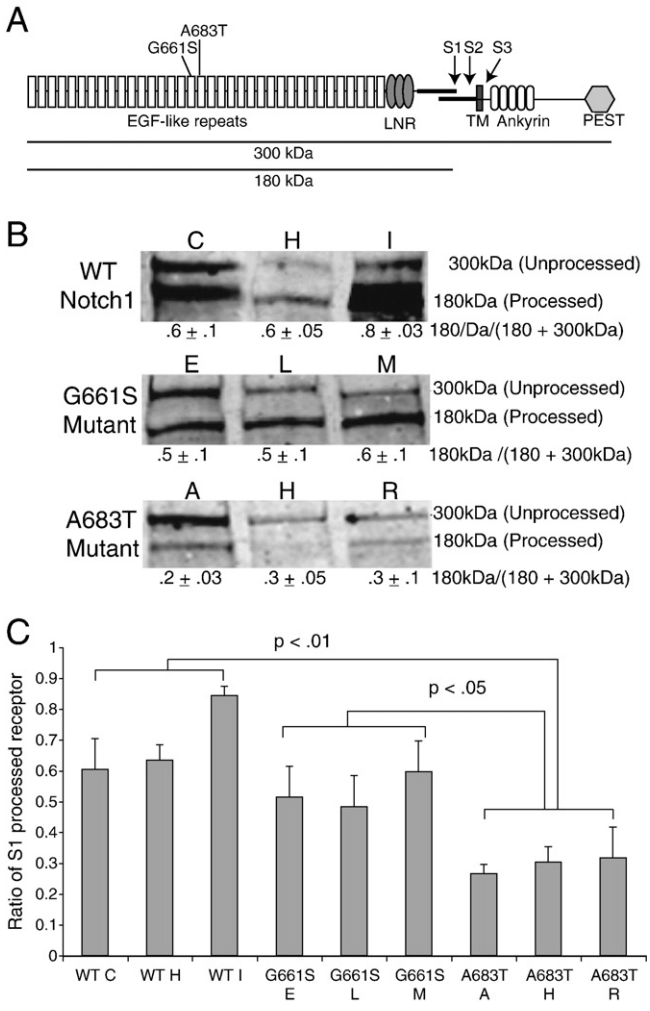

Fig. 1. Reduced S1-cleavage of NOTCH1^{A683T} receptor. A. Schematic showing the expected NOTCH1 products after typical processing events. The full length NOTCH1 protein (300 kDa) is cleaved at site 1 (S1) before presentation at the plasma membrane as a functional heterodimer (p180 and p120). Interaction with ligand triggers two subsequent cleavages (S2 and S3), releasing the intracellular domain (NICD) to activate downstream transcription factors. The LVOT-associated mutations (G661S, A683T) are located in the seventeenth and eighteenth EGF repeats in the extracellular domain (EC). B. Total protein lysates from three independent NIH3T3 cell lines stably expressing either N-terminally HA-tagged wild-type rNOTCH1 (clones C, H, I), rNOTCH1^{G661S} (clones E, L, M), or NOTCH1^{A683T} (clones A, H, R) receptor were analyzed by western blot using an anti-HA antibody. Note the decreased levels of the 180 kDa band (S1 Processed) in cells expressing the rNOTCH1^{A683T} receptor compared with wild-type rNOTCH1-expressing cells. Results from a representative experiment are shown. C. Band intensities were quantified and the percentage of total receptor that has undergone S1 cleavage was calculated (180 kDa/180 + 300 kDa). The average ratio of S1-cleaved protein is shown as the mean ± SD from three independent experiments. p-values were calculated using ANOVA followed by Bonferroni *post hoc*.

Recent research supports several important roles for Notch signaling during cardiac development. In the mouse, targeted deletion of *Notch1* or its nuclear partner RBPJK/CBF1/Su(H) results in impaired epithelial-to-mesenchymal transition (EMT) during cardiac cushion development, leading to a collapsed endocardium and the absence of cushion cells in the mesenchyme [13]. Combined loss of *Notch1* downstream targets, *Hey1* and *HeyL* also causes impaired EMT in mice [14]. EMT occurs in the endocardium around E9.0 to form the cardiac cushions, and is critical for proper outflow tract and atrioventricular canal development. During cardiac EMT, endocardial cells undergo significant changes in gene expression including Notch1-dependent induction of α-SMA, Snail1, and Snail2 [15,16]. In addition to its role in EMT, recent data indicates that Notch signaling plays additional critical roles in both the neural crest and the secondary heart field during development [17,18].

In this study we examine the impact of LVOT-associated *NOTCH1* mutations on Notch processing and induction of EMT. Two previously identified missense NOTCH1 variants that reduce JAGGED1 dependent Notch signaling are observed across a wide spectrum of LVOT phenotypes. One variant (NOTCH1^{G661S}) was present in patients with AVS, CoA, and HLHS, as well as in a patient with bicuspid aortic valve (BAV), reinforcing the idea of a common pathogenic mechanism. Interestingly, although these mutations are found at highly conserved sites within the NOTCH1 protein, alignment with other EGF repeats suggests that they might be well-tolerated substitutions. Indeed, all of these variants were also observed in an unaffected parent, so they can be tolerated in some developmental conditions. Given these conflicting findings, we examined the functional effects of these mutations on NOTCH1 protein maturation, trafficking and function, as well as their effects on the induction of EMT in endothelial cell lines, to determine how these missense variants might contribute to human disease.

2. Materials and methods

2.1. Cell culture and plasmids

NIH3T3 cells were cultured as previously described [9]. The HMEC-1 microvascular endothelial cell line was provided by the Centers for Disease Control and Prevention (Atlanta, Ga) and cultured as previously described [19]. Rat cDNAs encoding N-terminally HA-tagged rat NOTCH1, mutant NOTCH1^{G661S}, and mutant NOTCH1^{A683T} were described previously [9]. GenBank Accession numbers are NM_017617.3 for human NOTCH1 and NM_001105721.1 for rat NOTCH1. Protein numbering reflects the initiation codon as codon 1. To generate stable NOTCH1 cell lines, 4×10^4 NIH3T3 cells were plated in 24 well plates and transfected using Lipofectamine 2000 with 0.8 μg HA-tagged wild-type or mutant NOTCH1 expression vectors. Stable lines were generated by expanding individual colonies after culturing the cells for 15 days after transfection in the presence of G418 (0.6 mg/ml). NOTCH1 expression levels were determined by western blot. Cell lines exhibiting a range of NOTCH1 expression levels were expanded for use in further studies. Cell lines exhibiting similar levels of wild-type or mutant NOTCH1 were maintained to facilitate comparison between cell lines.

2.2. Western blot

1.5×10^5 NIH3T3 cells stably expressing NOTCH1, mutant NOTCH1^{G661S}, or mutant NOTCH1^{A683T} were plated in 6 well plates. After 24 h cells were lysed, run on a 6% SDS-PAGE gel, transferred to nitrocellulose, and NOTCH1 protein was detected with a mouse anti-HA antibody (HA-7, 1:1000, Sigma-Aldrich) and Alexafluor anti-mouse 688 secondary antibody (1:20,000, Invitrogen). Band intensity was quantified using Li-Cor Odyssey 2.1 software. All exposures were in the linear range, as determined by this software. p300 and p180 bands were quantified, and the % of total protein cleaved was calculated as p180/(p180 + p300). Each experiment was performed in triplicate and statistical analysis was performed (one way ANOVA, followed by Bonferroni *post hoc*). Western blots after co-culture assays were performed essentially as above, using the following primary antibodies: SNAIL 1(Cell Signaling Technologies #3895 1:1000), SNAIL 2 (Cell Signaling Technologies #9585 1:500), Hey2 (Millipore #AB15632 1:1000), HEYL (Millipore #MAB10094, 1:1000) and α-Tubulin (Sigma-Aldrich T5168 1:1000). In some cases, western blots were developed using ECL plus, following the manufacturers recommendations.