

RESEARCH ARTICLE

NOTCH3 Internal Tandem Duplication Defines a Novel Oncogenic Activation Mechanism of NOTCH Signaling

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ABSTRACT

NOTCH signaling is activated in tumors through multiple mechanisms, including mutations, gene rearrangements, and gene amplification. We report a novel activation mechanism, an internal tandem duplication (ITD) near the NOTCH3 negative regulatory region (NRR), found in a myogenic mesenchymal neoplasm. This 17-amino acid residue duplication disrupts the tightly autoinhibited structure surrounding the S2 cleavage site, resulting in ligand-independent S2 cleavage and constitutive pathway activation, as demonstrated by increased expression of the NOTCH3 target gene *HES1*. Cells expressing NOTCH3-ITD showed increased nuclear localization of the receptor and exhibited malignant phenotypes, including enhanced proliferation and migration. Together, these findings support the oncogenic role of NOTCH3-ITD.

1 | Introduction

The NOTCH signaling pathway is a highly conserved intercellular pathway extensively involved in regulating important biological processes such as cell proliferation, differentiation, apoptosis, and stem cell maintenance [1]. Both abnormal activation and loss-of-function of the NOTCH signaling pathway can contribute to tumorigenesis, and the functional outcomes depend on the specific genetic background and cellular environment [2]. Mechanisms leading to NOTCH pathway activation are diverse and include activating mutations in the receptor's negative regulatory region (NRR), nonsense/frameshift mutations at the C-terminal PEST domain, gene amplification, and fusion.

The spectrum of *NOTCH* genomic alterations varies significantly across different cancer types. For example, activating

mutations in the *NOTCH1* NRR domain are one of the primary driving events in T-cell acute lymphoblastic leukemia (T-ALL) [3], and in adenoid cystic carcinoma (AdCC) [4, 5]. *NOTCH1/2* PEST truncation mutations are observed in chronic lymphocytic leukemia (CLL) [6], mantle cell lymphoma (MCL) [7], diffuse large B-cell lymphoma (DLBCL) [8], and splenic marginal zone lymphoma (SMZL) [9]. Furthermore, *NOTCH* gene fusions are primarily observed in pericytic (perivascular) tumors [10] and some triple-negative breast cancers (TNBC) [11], while *NOTCH3* amplification is relatively common in high-grade serous ovarian cancer (HGSOC) [12].

Recently, internal tandem duplications (ITD) have gradually garnered attention as a novel mechanism of *NOTCH* activation. For example, ITD mutations in the *NOTCH1* juxtamembrane expansion (JME) domain promote the exposure of the S2 site and the release of the NOTCH intracellular domain (NICD), occurring

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in approximately 3%–5% of T-ALL cases [13]. Most recently, Ungureanu et al. reported the presence of *NOTCH3* ITD in two cases of myofibroma, a finding suggesting that ITD-mediated structural activation may not be limited to *NOTCH1* but may represent a potential common activation mechanism across the *NOTCH* receptor family [14]. Here we report a myogenic mesenchymal neoplasm harboring a previously undescribed somatic *NOTCH3*-ITD (Y1610_R1627 repeat within exon 26). Through a series of in vitro assays, we demonstrated that the ITD induces a structural alteration in the *NOTCH3* S2 region, resulting in ligand-independent S2 site exposure and constitutive activation of *NOTCH* signaling. This study elucidates the mechanism of abnormal *NOTCH3*-ITD activation and further expands the molecular spectrum of abnormal *NOTCH* signaling in mesenchymal tumors.

2 | Materials and Methods

2.1 | Immunohistochemistry

Immunohistochemical staining was performed on 5- μ m FFPE sections. The panel of antibodies included Vimentin, Desmin, Calponin, CD34, muscle-specific actin (MSA), smooth muscle actin (SMA), estrogen receptor (ER), progesterone receptor (PR), cytokeratin (CK), p63, SATB2, S-100, synaptophysin, MyoD1, and myogenin. Staining was carried out according to the following protocol: the slides were baked for 1 h at 60°C, deparaffinized, and rehydrated using 100% xylene, 100% ethanol, and running water, respectively. Antigen retrieval was performed by heating slides in citrate buffer (pH 6.0) or EDTA buffer (pH 9.0). The slides were blocked with a solution containing 10% normal serum and 1% bovine serum albumin (BSA) in Tris-buffered saline, followed by incubation with primary antibodies for 2 h at room temperature or overnight at 4°C. Endogenous peroxidase activity was inhibited with 0.3% hydrogen peroxide, and slides were incubated with a horseradish peroxidase-labeled polymer (DAKO). The tissue sections were developed using 3,3'-diaminobenzidine (DAKO) as the chromogen and counterstained with Mayer's hematoxylin.

2.2 | Targeted DNA Next-Generation Sequencing

Genomic DNA was isolated from FFPE tumor tissue sections and matched peripheral blood samples using QIAamp DNA Micro Kits (Qiagen, Germany). Three hundred nanograms of DNA were fragmented to a size of 200–300 bp with a Bioruptor Pico (Diagenode, New Jersey, USA). Library preparation was performed using the Rapid Plus DNA Lib Prep Kit for Illumina (ABclonal) according to the manufacturer's instructions. The libraries were hybridized with a pool of biotin-labeled bait oligos targeting 638 cancer-related genes and genome-wide SNP loci for 16 h. Targeted regions were captured with streptavidin beads, followed by PCR amplification, and sequenced as paired-end 150-bp reads on an Illumina NextSeq 6000 instrument. Reads were aligned to the reference genome (hg19) using BWA-MEM. Genome Analysis Toolkit (GATK) was used to mark the duplicates and perform base quality score recalibration. Coverage and sequencing statistics were determined using Bamdst and in-house script. Single nucleotide variants and small insertion/

deletion mutations were called with VarDict and annotated with Annovar. Raw vcf files were filtered by general population frequency in several databases (1000 genomes, GnomAD etc.), allowing only variants with minor allele frequencies < 1% to keep. In addition, variation with an allelic fraction less than 5% was removed. Structure variation analysis was completed by Seeksv. MSIsensor was used for MSI analysis. Copy number variation was analyzed by in-house software. To ensure high-confidence variant calling, rigorous quality control (QC) criteria were applied to the sequencing data. The minimum acceptable thresholds were defined as follows: average sequencing depth $\geq 500\times$, coverage uniformity $\geq 90\%$, sequence mapping rate $\geq 90\%$, and Q30 base percentage $\geq 80\%$.

2.3 | Whole Transcriptome Sequencing

Total RNA from FFPE tumor tissue sections was extracted with TRIZOL reagent according to the manufacturer's instructions (Cat: 10296010, ThermoFisher, Invitrogen, USA). RNA quantity and quality were assessed, and library preparation was performed using the VAHTS Universal V8 RNA-seq Library Prep Kit for Illumina (Cat: NR605, Vazyme, China) following the manufacturer's protocol. The workflow included RNA fragmentation, first- and second-strand cDNA synthesis, end repair, A-tailing, and adaptor ligation. The resulting libraries were amplified and sequenced on a NovaSeq 6000 platform (Illumina, USA) to generate paired-end 150-bp reads. Sequencing reads were aligned to human genome with STAR. Fusions were called with STAR-Fusion v1.10, Arriba v2.1, and FusionCatcher v1.33 integrated in the SANO Fusion Pipeline; only in-frame events supported by ≥ 10 reads and ≥ 0.1 FPKM were retained.

2.4 | Cell Culture

Human embryonic kidney 293T (HEK293T) cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator with 5% CO₂. Cells were passaged every 2–3 days to maintain logarithmic growth. Mycoplasma contamination was routinely tested before experiments, and only cells testing negative were used for subsequent experiments.

2.5 | Lentivirus Production and Infection and Transient Transfection

NOTCH3 wild-type (*NOTCH3*-WT), *NOTCH3* internal tandem duplication mutant (*NOTCH3*-ITD), and *ACTB*::*NOTCH3* fusion protein expression plasmids were constructed using standard molecular cloning techniques. All gene sequences were cloned into the pcDNA vector with a C-terminal Flag tag and driven by the CMV promoter. The *NOTCH3*-ITD construct was generated by site-directed mutagenesis of the wild-type sequence, and the *ACTB*::*NOTCH3* fusion plasmid was obtained via PCR amplification and ligation. All constructs were sequence-verified by Sanger sequencing.

Transient transfection of the pcDNA-Flag expression plasmids into 293T cells was performed using Lipofectamine 3000

according to the manufacturer's instructions. The empty pcDNA vector served as a negative control. Cells were collected 24–48 h posttransfection for downstream functional and mechanistic assays.

2.6 | Cell Counting Kit-8 (CCK-8) Assay

Cell proliferation was assessed using the CCK-8 assay. Transfected 293T cells were seeded at equal densities in 96-well plates. At indicated time points (0, 24, 48, and 72 h), CCK-8 reagent was added, and absorbance at 450 nm was measured using a microplate reader.

2.7 | Wound Healing Assay

Cell migration was evaluated by wound healing assay. Transfected 293T cells were grown to near confluence, and a linear scratch was made using a sterile pipette tip. Detached cells were removed by washing with PBS, and cells were incubated in serum-free medium. Images were captured at 0, 24, and 48 h, and wound closure was quantified using ImageJ software.

2.8 | Immunofluorescence Imaging

Transfected cells were seeded on coverslips, fixed with 4% paraformaldehyde, permeabilized with Triton X-100, and blocked. Cells were incubated with anti-Flag primary antibody, followed by fluorescently labeled secondary antibody. Nuclei were counterstained with DAPI, and images were acquired using a fluorescence microscope.

2.9 | qRT-PCR

Total RNA was extracted from cells using a commercial RNA extraction kit (TianGen, China) and reverse-transcribed into cDNA. Quantitative PCR (qPCR) was performed using SYBR Green to determine the mRNA expression levels of *NOTCH3-WT*, *NOTCH3-ITD*, and *ACTB::NOTCH3*. The primers used were as follows:

NOTCH3-WT/NOTCH3-ITD: Forward 5'-TGGAGATTGACAACCGGCTC-3'; Reverse 5'-ACGAGAATGACCAGCAGCAA-3'
ACTB::NOTCH3: Forward 5'-CTTCAGTTGGCATTGGCTCC-3'; Reverse 5'-AGGCATCCTCACCTGAAGT-3'.

18S rRNA was used as the internal reference gene. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method.

2.10 | Western Blot Analysis

Total protein was extracted using RIPA lysis buffer, and protein concentration was determined by BCA assay. Equal amounts of protein were separated by SDS-PAGE, transferred to PVDF membranes, blocked, and incubated with primary and HRP-conjugated secondary antibodies. Protein signals were detected using chemiluminescence and quantified.

2.11 | Statistical Analysis

All experiments were performed with at least two independent replicates. Data are presented as mean $\pm$ standard deviation (SD). Statistical analyses were conducted using GraphPad Prism software (version 9.3, GraphPad Software, San Diego, California, USA). Comparisons between two groups were performed using Student's *t*-test, while multiple group comparisons were conducted using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A *p*-value of less than 0.05 was considered statistically significant.

3 | Results

3.1 | Clinical Information

The patient is a 9-year-old female who presented with a vulvar mass located near the clitoris, first noted approximately 1 year ago. The mass was soft in consistency, measured approximately $3 \times 2 \times 1$ cm, and was mobile, without associated erythema, swelling, or pain. Microscopically, tumor cells grew in a nodular pattern within a myxoid background. Tumor cells were arranged in a diffuse sheet-like pattern or in tubular or pseudo-glandular structures. The cells were small and round to oval, with scant cytoplasm and hyperchromatic nuclei (Figure 1A–D). Immunohistochemically, the tumor cells were positive for Desmin and Calponin (Figure 1E,F), and negative for estrogen receptor (ER), progesterone receptor (PR), cytokeratin (CK), p63, SATB2, S-100, synaptophysin, MyoD1, myogenin, CD34 (Figure 1G), muscle-specific actin (MSA; Figure 1H), and smooth muscle actin (SMA; Figure 1I). The patient underwent surgical resection only; notably, there has been no evidence of recurrence or metastasis in the 2 years since surgery.

3.2 | Genomic Profiling

Tumor DNA was analyzed using a targeted DNA next-generation sequencing (NGS) panel covering 638 cancer-related genes and genome-wide SNP loci. This analysis identified a 51-bp ITD in exon 26 of *NOTCH3* (Y1610_R1627) (VAF 41%), resulting in the insertion of 17 additional amino acids proximal to the S2 cleavage site (Figure 1J). In addition, transcriptome analysis of tumor RNA confirmed the presence of the ITD at the mRNA level (Figure 1K). No other genomic alterations, including large-scale chromosomal abnormalities, were detected (Figure 1L). Concurrent DNA NGS analysis of peripheral blood did not reveal the ITD, confirming its somatic origin.

3.3 | Cellular Localization of NOTCH3-ITD and Its Functional Consequences

To investigate the oncogenic potential of NOTCH3-ITD, we established an in vitro cell model by transfecting HEK293T cells with plasmids expressing FLAG-tagged NOTCH3-ITD, wild-type NOTCH3, or an empty vector control. As a positive control, we used a construct expressing *ACTB::NOTCH3*, a fusion previously identified in a glomus tumor. Expression of NOTCH3 and its mutants was confirmed by both qPCR and

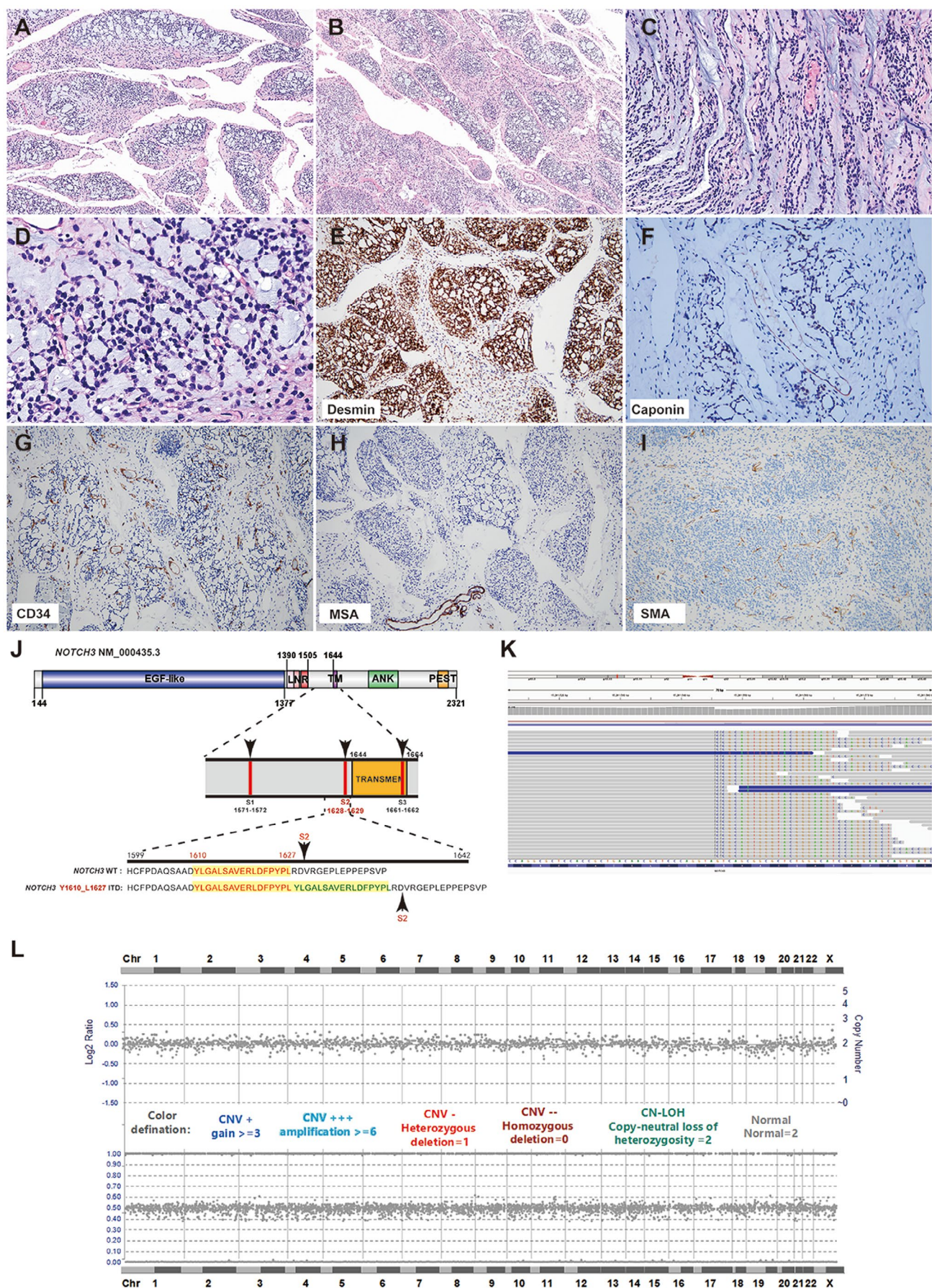


FIGURE 1 | Legend on next page.

FIGURE 1 | Histopathological and molecular features of the tumor. (A, B) Low-power view (10×) of H&E staining shows a nodular tumor architecture with a myxoid background. (C) Medium-power view (20×) reveals tumor cells arranged in reticular structures. (D) High-power view (40×) demonstrates small, round tumor cells with hyperchromatic, round-to-oval nuclei. Mitotic figures are rare. (E–I) Immunohistochemistry shows that tumor cells are positive for Desmin (E), Calponin (F), and negative for CD34 (G), muscle-specific Actin (MSA) (H), and smooth muscle Actin (SMA) (I). (J) Targeted DNA NGS identifies an internal tandem duplication (ITD) in *NOTCH3* exon 26 (p.Y1610_R1627dup). A schematic illustrates the domain structure of wild-type NOTCH3 and the location of the ITD. ANK, ankyrin repeat domain; EGF-like, epidermal growth factor-like domain; LNR, Lin12-NOTCH repeat; PEST, Proline, Glutamic acid, Serine, and Threonine-rich domain; TM, transmembrane domain. (K) Integrative Genomics Viewer (IGV) visualization showing transcription of the *NOTCH3*-ITD at the mRNA level. (L) Targeted DNA NGS shows no genome-wide copy number variations (CNVs).

Western blot analyses (Figure 2A). Subcellular localization was evaluated by immunofluorescence staining using an anti-FLAG antibody. Wild-type NOTCH3 localized predominantly to the cytoplasm and cell membrane, as expected, whereas both NOTCH3-ITD and ACTB::NOTCH3 showed cytoplasmic localization with a substantial fraction detected in the nucleus (Figure 2B). In wound-healing assays, cells expressing NOTCH3-ITD, wild-type NOTCH3, or ACTB::NOTCH3 all exhibited increased migratory capacity compared with vector control cells at both 24 and 48 h. At 48 h, cells expressing NOTCH3-ITD and ACTB::NOTCH3 migrated significantly faster than those expressing wild-type NOTCH3, with ACTB::NOTCH3-expressing cells showing the greatest migration (Figure 2C,D). Cell proliferation was assessed using the CCK-8 assay, which demonstrated that expression of all three NOTCH3 constructs significantly increased proliferation compared with empty vector controls. Notably, cells expressing NOTCH3-ITD and ACTB::NOTCH3 proliferated more rapidly than those expressing wild-type NOTCH3 (Figure 2E). To confirm the activation of downstream signaling, we assessed the expression of the canonical NOTCH target gene, *HES1*. Both quantitative real-time PCR (qRT-PCR) and Western blot analyses demonstrated a significant upregulation of *HES1* at both the mRNA and protein levels in cells expressing NOTCH3-ITD compared to control cells (Figure 2F,G).

3.4 | Protein Structure of Wild-Type NOTCH3 and NOTCH3-ITD

The ITD (Y1610_R1627) is located within the Negative Regulatory Region (NRR) of NOTCH3, which contains the S2 cleavage site and plays a critical role in NOTCH activation. To investigate the structural mechanism underlying ITD-driven activation, the NRR structures of wild-type NOTCH3 and NOTCH3-ITD were compared using AlphaFold. The annotation of key secondary structural elements, including the $\alpha 3$ helix and the $\beta 1$ and $\beta 5$ strands, was guided by the domain architecture established in a previous homology model derived from the NOTCH2 NRR crystal structure (PDB: 2004). Our model of the wild-type NRR reveals a compact, auto-inhibited conformation (Figure 3A). The interpretation of this structure's protective mechanism is strongly supported by published hydrogen-deuterium exchange mass spectrometry (HDX-MS) data on the NRR's solution dynamics (17). In this model, the S2 cleavage site (marked yellow) on the $\beta 5$ strand is sterically masked by a structural core formed by the antiparallel $\beta 1/\beta 5$ sheet and the tightly packed $\alpha 3$ helix. This visualization aligns perfectly with HDX-MS findings that peptides

from this core are highly protected from solvent exchange, indicating a stable yet dynamic shield. In contrast, the model of the NOTCH3-ITD mutant demonstrates a catastrophic failure of this protective mechanism (Figure 3B). The inserted ITD sequence (marked red) acts as a molecular wedge, completely disrupting the native packing of the $\alpha 3$ helix against the β -sheet. This forces the $\beta 5$ strand containing the S2 site into a permanently solvent-exposed state. This gross conformational rearrangement provides a clear structural basis for bypassing the regulated, dynamic unmasking of the S2 site, thereby explaining the constitutive cleavage and oncogenic activity of the NOTCH3-ITD mutant.

4 | Discussion

NOTCH signaling begins with S1 site cleavage, which occurs in the Golgi where a furin-like convertase cuts the newly synthesized NOTCH receptor into two noncovalently associated subunits that are then transported to the cell surface. Upon interacting with its ligand, NOTCH receptors undergo a conformational change that exposes the S2 cleavage site, which is cut by an ADAM metalloprotease outside the plasma membrane. This is followed by S3 cleavage within the transmembrane domain by γ -secretase, releasing the NOTCH intracellular domain (NICD), which translocates to the nucleus, interacts with the CSL transcription factor, and activates transcription of NOTCH target genes such as *HES* and *HEY* [15].

NOTCH signaling is activated by several mechanisms that lead to ligand-independent signaling in tumors. Mutations in the heterodimerization (HD) domain of NOTCH receptors destabilize the S2 site, allowing cleavage without ligand binding, while PEST domain mutations prevent degradation of the NICD, prolonging its nuclear activity [3]. Overexpression or amplification of NOTCH receptors or ligands (Delta/Jagged) in tumor cells or the surrounding stroma can drive sustained pathway activation, and increased γ -secretase activity can also enhance NICD release [16]. Together, these alterations increase transcription of NOTCH target genes, promoting proliferation, survival, and tumor progression. Interestingly, in glomus tumors, NOTCH signaling is mostly activated by NOTCH rearrangements; for example, NOTCH is fused to partners such as *MIR143* or *CARMN* [17]. These fusions place the strong *MIR143* or *CARMN* promoter next to the 3' end NOTCH encoding the NICD. The resulting truncated protein resembles the active NICD that enters the nucleus and drives NOTCH target gene transcription, effectively activating the pathway and contributing to tumor development. Among NOTCH1–4, NOTCH2 is the most frequently rearranged

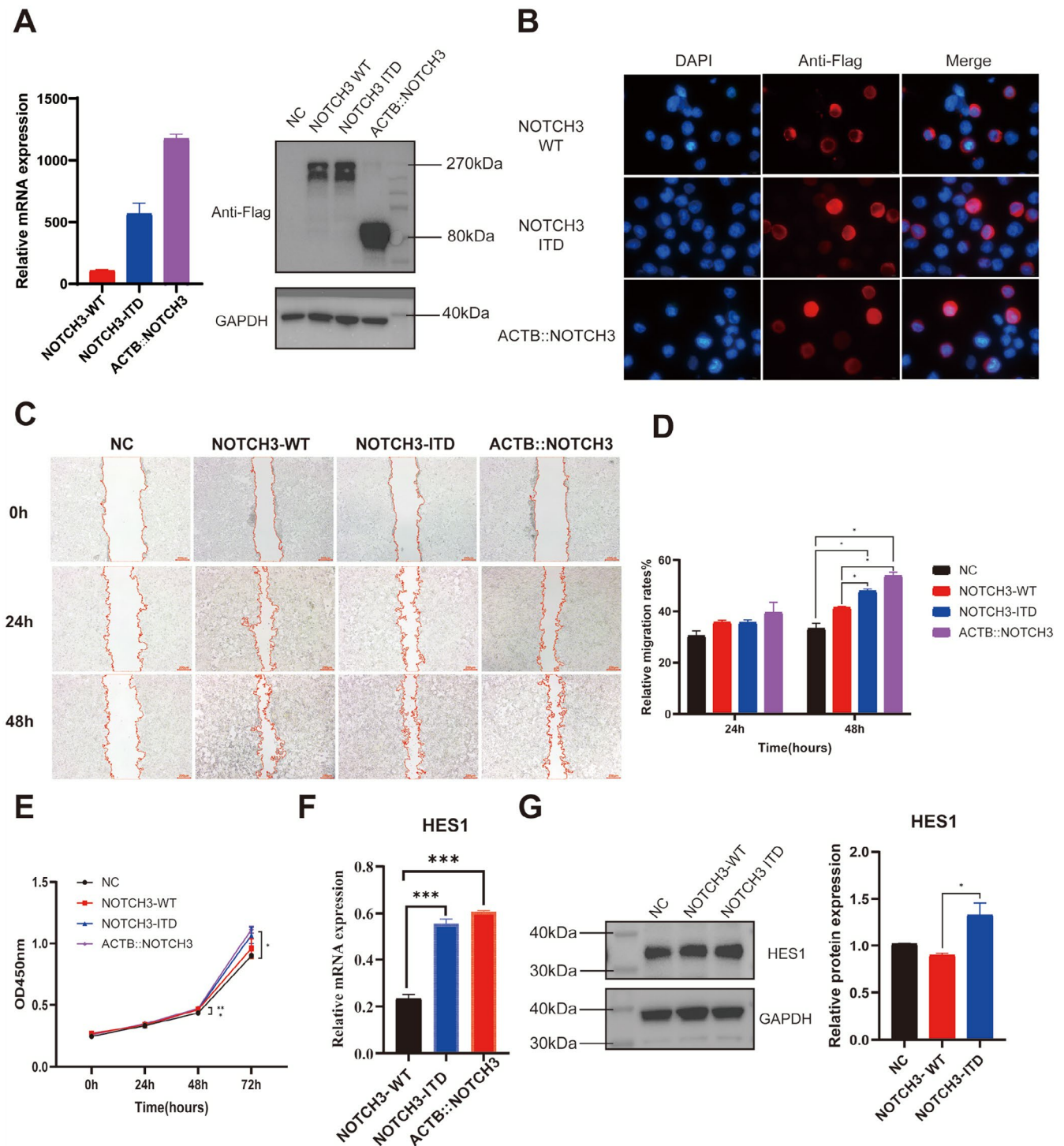


FIGURE 2 | Functional characterization of the NOTCH3-ITD mutation. (A) HEK293T cells were transiently transfected with plasmids encoding Flag-tagged wild-type NOTCH3 (NOTCH3-WT), NOTCH3-ITD, ACTB::NOTCH3 (positive control), or an empty vector (negative control). Transfection efficiency was verified by qPCR and Western blot analyses. (B) Immunofluorescence staining shows increased nuclear localization of NOTCH3-ITD and ACTB::NOTCH3 proteins detected by FLAG-specific antibody (red). Nuclei were counterstained with DAPI (blue). (C, D, E) Cells expressing NOTCH3-WT, NOTCH3-ITD, or ACTB::NOTCH3 showed increased migratory capacity and proliferation compared to vector control cells. NOTCH3-ITD and ACTB::NOTCH3-expressing cells demonstrated significantly greater migration and proliferation than NOTCH3-WT-expressing cells, with ACTB::NOTCH3 producing the strongest effect. Data are presented as the mean $\pm$ SD ($n=2$); statistical significance is indicated as $p < 0.05$ (*) and $p < 0.001$ (***). (F, G) Expression of the NOTCH target gene HES1 was significantly upregulated at both the mRNA level, measured by qRT-PCR (F), and the protein level, measured by Western blot and quantified using ImageJ software (G), in cells expressing NOTCH3-ITD. Data are presented as the mean $\pm$ SD ($n=2$); statistical significance is indicated as $p < 0.05$ (*).

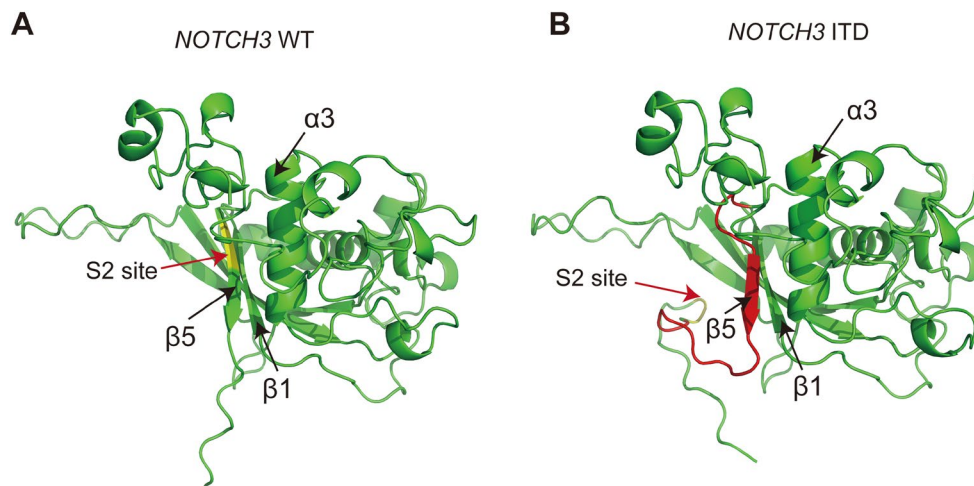


FIGURE 3 | Structural models of the negative regulatory region (NRR) of wild-type NOTCH3 and NOTCH3-ITD. (A) The predicted structure of wild-type NOTCH3 NRR. Annotation of secondary structural elements (e.g., $\alpha 3$ helix, $\beta 1/\beta 5$ strands) is based on a prior homology model. The S2 cleavage site (marked yellow) is shielded by a protective core. (B) The predicted structure of the NOTCH3-ITD mutant. The ITD sequence is marked red. Its presence disrupts the protective core and induces a major conformational rearrangement, resulting in constitutive solvent exposure of the S2 cleavage site.

in glomus tumors, whereas *NOTCH4* alterations have not been documented to date.

We describe a novel mechanism of NOTCH3 activation via an ITD that spans amino acids Y1610–R1627 at the S2 site, which is normally buried within the NRR of the receptor. In the wild-type NOTCH3 receptor, ligand binding induces a conformational change and mechanical pulling that exposes S2, enabling ADAM protease access and NOTCH signaling. The NOTCH3-ITD disrupts the autoinhibitory structure by introducing extra residues into the NRR domain, causing steric strain and local unfolding that exposes S2 independently of ligand binding. This allows ligand-independent ADAM-mediated S2 cleavage, followed by γ -secretase-mediated S3 cleavage, releasing the NOTCH3 NICD into the nucleus and driving constitutive transcription of NOTCH target genes. Although the precise transcriptomic changes have not yet been evaluated, they evidently drive increased cell proliferation and migration in vitro. However, it is noteworthy that the promotive effects of the NOTCH3-ITD on cellular proliferation and migration were significantly milder than those induced by the *ACTB::NOTCH3* fusion (Figure 2C–F). This intermediate in vitro phenotype suggests that the ITD mutation confers a relatively low degree of malignancy. Importantly, this finding perfectly aligns with the patient's indolent clinical course; following surgical excision alone, without adjuvant chemotherapy or radiotherapy, she has remained free of recurrence and metastasis for over 2 years.

ITD is a type of genomic mutation in which a segment of DNA within a gene is duplicated and inserted immediately adjacent to the original sequence. ITDs often arise from replication errors in DNA regions containing direct repeats or palindromic sequences, where misalignment during DNA replication leads to duplication. When ITDs occur in regulatory or autoinhibitory regions of a protein, they can disrupt normal structural constraints and cause ligand-independent constitutive activation. A well-known example is the FLT3-ITD in acute myeloid leukemia (AML), which occurs in the juxtamembrane domain and

leads to constitutive kinase activation; FLT3-ITDs are observed in approximately one-third of AML cases [18].

In most oncogenic NOTCH fusions, the fusion protein contains the functional NICD domain with the S2 and S3 sites lost due to gene rearrangement. This leads to constitutive activation of NOTCH signaling, bypassing the normal ligand-dependent S2 and S3 cleavage steps. However, rare fusions, such as *CARMN::NOTCH1/2*, retain an intact S3 region and remain dependent on downstream γ -secretase (S3) cleavage, showing sensitivity to γ -secretase inhibitors (GSIs). For example, Zhang et al. [19] reported a pediatric patient with metastatic glomus tumor harboring a *CARMN::NOTCH1* fusion who achieved significant and durable disease control following GSI therapy. In the case of NOTCH3-ITD, the S3 region is intact, so NICD release still requires γ -secretase cleavage, suggesting that patients with NOTCH3-ITDs may also be sensitive to GSIs as a therapeutic strategy.

The histopathological diagnosis in this patient is challenging. The initial differential diagnosis included angiomyofibroblastoma; however, angiomyofibroblastoma is a hormone-driven tumor and typically expresses ER/PR, both of which were negative in this case. Molecular analysis of our case identified a NOTCH3-ITD, which leads to NOTCH pathway activation. Although NOTCH3 activation is a well-recognized driver of pericytic and glomus differentiation, the histology and immune profile of this tumor are incompatible with a classic glomus tumor. Typical glomus tumors occur in the extremities, most classically in the subungual region; vulvar involvement is rare but has been documented. Immunohistochemically, glomus tumors are often positive for SMA and negative for desmin. However, our tumor showed desmin positivity and SMA negativity. Taken together, we provisionally classify this tumor as a myogenic mesenchymal neoplasm with NOTCH3-ITD.

In summary, this study identified a novel NOTCH3-ITD in a myogenic mesenchymal neoplasm. Functional analyses

demonstrated that the NOTCH3-ITD localizes predominantly to the nucleus instead of the cell membrane, and cells expressing the NOTCH3-ITD exhibited increased proliferation and migration, confirming its transforming capability. Importantly, this alteration retains the S3 cleavage site, suggesting potential sensitivity to GSIs and providing a possible therapeutic approach.

Author Contributions

R.M. and S.X. designed the project. H.G. and S.C. drafted the manuscript. H.G., Y.W., J.F., and R.M. performed pathological diagnoses and collected clinical and pathological data. S.C., M.Y., Y.L., Z.B., H.C., J.F., and L.M. conducted molecular analyses. All authors contributed to data interpretation and participated in discussions of the results.

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The authors have nothing to report.

Ethics Statement

This study was conducted on retrospectively collected, anonymized patient samples and associated clinical data. All procedures were carried out in accordance with institutional guidelines and the principles outlined in the Declaration of Helsinki. Identifiable personal information was not included in the analysis.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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