

# Molecular dynamic simulation of mutated $\beta$ -catenin in solid pseudopapillary neoplasia of the pancreas

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**Abstract.** Solid pseudopapillary neoplasia of the pancreas (SPN) is a rare pancreatic neoplasm that frequently harbors mutations in catenin  $\beta$ 1 (*CTNNB1*, encoding  $\beta$ -catenin) as a part of its molecular pathogenesis. Mutations to *CTNNB1* reported in SPN usually occur at the serine/threonine phosphorylation sites, including codons 33, 37 and 41, and the flanking residues of codon 33. On analysis of 3 cases of SPN, mutations to *CTNNB1* were detected in codon 32 (D32A and D32Y). As this residue, aspartic acid, is not a direct phosphorylation site of the protein, molecular modeling tools were used to predict the influence of these mutations on the protein structure of  $\beta$ -catenin. A total of three MD simulations (wild-type, D32A, and D32Y) were performed to visualize the conformations of  $\beta$ -catenin under *in vivo*, aqueous-phase conditions at 37°C. In the wild-type protein, the secondary structure of residues P16-H28 remained helical; we therefore hypothesized that the helical structure of this protein fragment (residues M11-G50) was necessary for phosphorylation of S33 phosphorylation. The loss of the secondary structure in P16-H28 was observed in D32A, losing its helical structure and becoming a turn; however, in the D32Y mutant, the helical structure remained. The present demonstrated that structural changes in the mutated  $\beta$ -catenin protein at D32 could potentially explain the mechanism behind its defective phosphorylation in the pathogenesis of SPN.

## Introduction

Solid pseudopapillary neoplasia of the pancreas (SPN) is a rare pancreatic neoplasm accounting for <2% of pancreatic exocrine tumors. SPN usually occurs in young female patients

during the second to third decade of life (1). In the majority of cases, the patient presents with a mixed cystic-solid mass in any region of the pancreas, which may compress adjacent organs and cause symptoms (2). SPN is generally regarded as a low-grade malignant tumor that is confined to the pancreas, for which en-bloc resection is the primary therapeutic method (3). The prognosis is generally favorable, except for patients with invasion and metastasis at the time of diagnosis, which is reported in ~5% of cases (4,5).

Histopathologically, SPN displays solid pseudopapillary areas intermixed with cystic regions, and consist of uniform polygonal-shaped cells with oval nuclei and abundant cytoplasm, with occasional pleomorphism and mitosis (6). The majority of SPNs are marked with neuron-specific enolase, vimentin and  $\alpha$ 1-antitrypsin and are immunohistochemically negative for cytokeratin AE1/AE3 (3). In addition, Ki-67 immunoreactivity is associated with tumor aggressiveness and poorer patient prognosis. A recent study has revealed that the nuclear accumulation of the  $\beta$ -catenin protein is one of the pathological hallmarks of SPN (7). Further investigations reported somatic mutations to catenin  $\beta$ 1 (*CTNNB1*) in the tumor tissue, with the majority being point mutations at serine/threonine residues that are phosphorylation sites of glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), including at codons 33, 37 and 41, and the flanking residues of codon 33 (codons 32 and 34) (8). The most common mutation was reported at codon 32 (8).

$\beta$ -catenin, encoded by *CTNNB1*, is centrally involved in the Wnt signaling pathway, which serves physiological roles in embryonic organ development (9). In differentiated cells, the cytosolic level of  $\beta$ -catenin is kept low by a ubiquitination process driven by phosphorylation of  $\beta$ -catenin at its clustered serine/threonine residues in exon 3 by casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3), which are the scaffolding proteins that serve a role in  $\beta$ -catenin ubiquitination and proteasomal degradation (9). Mutations involving these phosphorylation sites are involved in the molecular pathogenesis of various pediatric embryonal tumors, including hepatoblastoma, Wilms tumor, medulloblastoma and pancreaticoblastoma (10-12). Codon 32, encoding for aspartic acid, is not the phosphorylation target itself. However, point mutations at Asp32 have been reported in various types of human cancer, including SPN (8). The juxtaposition of Asp32 mutation with Ser33 may result in conformational changes that prevent

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effective phosphorylation, hence the cytosolic retention of  $\beta$ -catenin and its translocation into the nucleus (13).

Functional genetic studies focusing on codon 32 revealed a reduction in ubiquitination activity in D32G, D32N and D32Y mutants when compared to the wild-type sequence (13,14). However, details regarding structural changes caused by codon 32 mutations have not been pursued. The present study investigated mutations to *CTNNB1* detected in samples from three patients SPN and used molecular dynamics (MD) simulation to attempt to predict alterations to protein conformations caused by the mutations.

## Materials and methods

**Samples and polymerase chain reaction.** The present study was approved by the Research Ethics Committee of the Faculty of Medicine, Prince of Songkla University (Hat Yai, Thailand) and patients provided written informed consent agreeing to their inclusion. Snap-frozen tumor specimens from three patients with SPN that underwent surgical resection in Songklanagarind Hospital were retrieved for DNA extraction. The cases included 1 male and 2 females, aged 12, 13 and 61 years, respectively. DNA extraction was done using GeneJET genomic DNA purification kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA), following manufacturer's protocol. A mutation study covering the exon 2-4 of *CTNNB1* was performed by polymerase chain reaction and direct nucleotide sequencing using 2 primer sets designed by Koch *et al* (15) and the PCR conditions reported by the study of Udatsu *et al* (16). PCR polymerase was performed by using TopTaq Master Mix kit (Qiagen, Hilden, Germany) with the condition as follows: 5 min at 95°C, 30 cycles (30 sec at 95°C, 30 sec at 58°C, 45 sec at 72°C) and 10 min at 72°C. All amplicon was then purified by GeneJET PCR Purification kit (Thermo scientific, Massachusetts, USA). Nucleotide sequencing was performed by the Scientific Equipment Center, Prince of Songkla University. Mutations to *CTNNB1* in each case involved codon 32, consisting of two incidences of D32A and one of D32Y (Table I).

**Immunohistochemistry.** Immunohistochemistry of the tumor tissue used the protocol presented in our previous work (17). Sections of 3- $\mu$ m thickness were stained using  $\beta$ -catenin monoclonal antibody (cat. no. 6B3; BD Biosciences, Franklin Lakes, NJ, USA) at a 1:1,000 dilution. Detection was then performed by using Bond Polymer Refine Detection system (Leica, Ltd., Milton Keynes, UK). This included all reagents: Peroxide blocking reagent (3-4%), polymer anti-mouse poly-HRP-IgG (<25  $\mu$ g/ml) containing 10% (v/v) animal serum in tris-buffered saline/0.09% ProClin™ 950, DAB Part 1 (66 mM 3,3'-diaminobenzidine tetrahydrochloride hydrate, in a stabilizer solution), DAB Part B ( $\leq$ 0.1% (v/v) hydrogen peroxide in a stabilizer solution), and hematoxylin. The protocol used in this study was performed as followed: After endogenous peroxidase blocking, slides were incubated at room temperature with primary antibody for 120 min, followed by a 30 min incubation at room temperature with peroxidase labeled polymer conjugated to rabbit anti-mouse immunoglobulins. Color was then developed by the liquid 3,3'-diaminobenzidine tetrahydrochloride hydrate (DAB)

chromogen. Counterstaining was performed with hematoxylin and imaged using a light microscope (magnification, x20 and x40). All procedures were performed according to the Bond Polymer Refine Detection system manufacturer protocol. Nuclear accumulation of  $\beta$ -catenin was demonstrated in the tissue of all cases (Fig. 1).

### *MD simulation of wild-type and mutated forms of $\beta$ -catenin.*

To elucidate the role of the aforementioned mutations on  $\beta$ -catenin function, a three-dimensional (3D) structure was required. The wild-type  $\beta$ -catenin template was adopted from the first structure of an experimental nuclear magnetic resonance-derived structure from a rabbit (pdb code, 2G57), in which the sequence identity in the region of interest is identical to its human counterpart. As mutant structures of human  $\beta$ -catenin are not available, bioinformatics tools were introduced in the present study. In addition, MD simulation was performed to investigate the effects of point mutations on the protein conformation in detail.

The structure prediction was commenced using residues 11-50 of the chosen  $\beta$ -catenin sequence. The residue numbers refer to those of the human  $\beta$ -catenin protein sequence (<http://www.uniprot.org/uniprot/P35222>). For each  $\beta$ -catenin (wild-type, D32A or D32Y), a secondary structure was independently predicted using two bioinformatics tools, PSIPRED Protein Sequence Analysis Workbench (18,19) and NetSurfP version 1.1 (20). A prediction of the 3D structure of the wild type was performed using PEP-fold server (21-23), using the structure of rabbit  $\beta$ -catenin as a molecular template. The wild-type structure was finally chosen from 50 possible structures corresponding to the aforementioned predicted secondary structure predictions. For the mutant, the 3D structures of D32A and D32Y were constructed using the point mutation module of the Rosetta Backrub server (23), and the aforementioned wild-type structure was exploited as a template. The selection of the predicted structure was performed using the best result from 50 possible structures based on the highest prediction score.

Each constructed  $\beta$ -catenin structure was solvated in the TIP3P water rectangular box,  $\sim$ 4,400 water molecules, with a distance of 12 Å from the protein surface using the command from tleap module in AMBER16 package. Sodium chloride was then added into the system to make it equivalent to 0.15 M NaCl solution. Protonation states in all ionizable amino acid side chains were set at pH 7. The protein-solution system was modeled using AMBER10 force field, which was included in an AMBER 16 package (24). Prior to MD simulation, the system was energy-minimized to remove unusual inter-atomic contacts, using the steepest descent method for 2,000 steps. The system was then equilibrated in a constant number (N), volume (V), and temperature (T) (NVT) ensemble for 600 psec, where the protein was position-restrained using force constants of 250, 150, 100, 50, 20, and 10 kcal/mol/Å<sup>2</sup> for each 100 psec simulation. A time step of 1 sec was applied in each NVT run and a temperature of 310 K (37°C) was controlled using Langevin dynamics (25). The simulation was subsequently switched to an isobar/isothermal (constant number (N), pressure (P), and temperature (T); NPT) ensemble, with a temperature of 310 K and a pressure of 1 atm, regulated by Berendsen





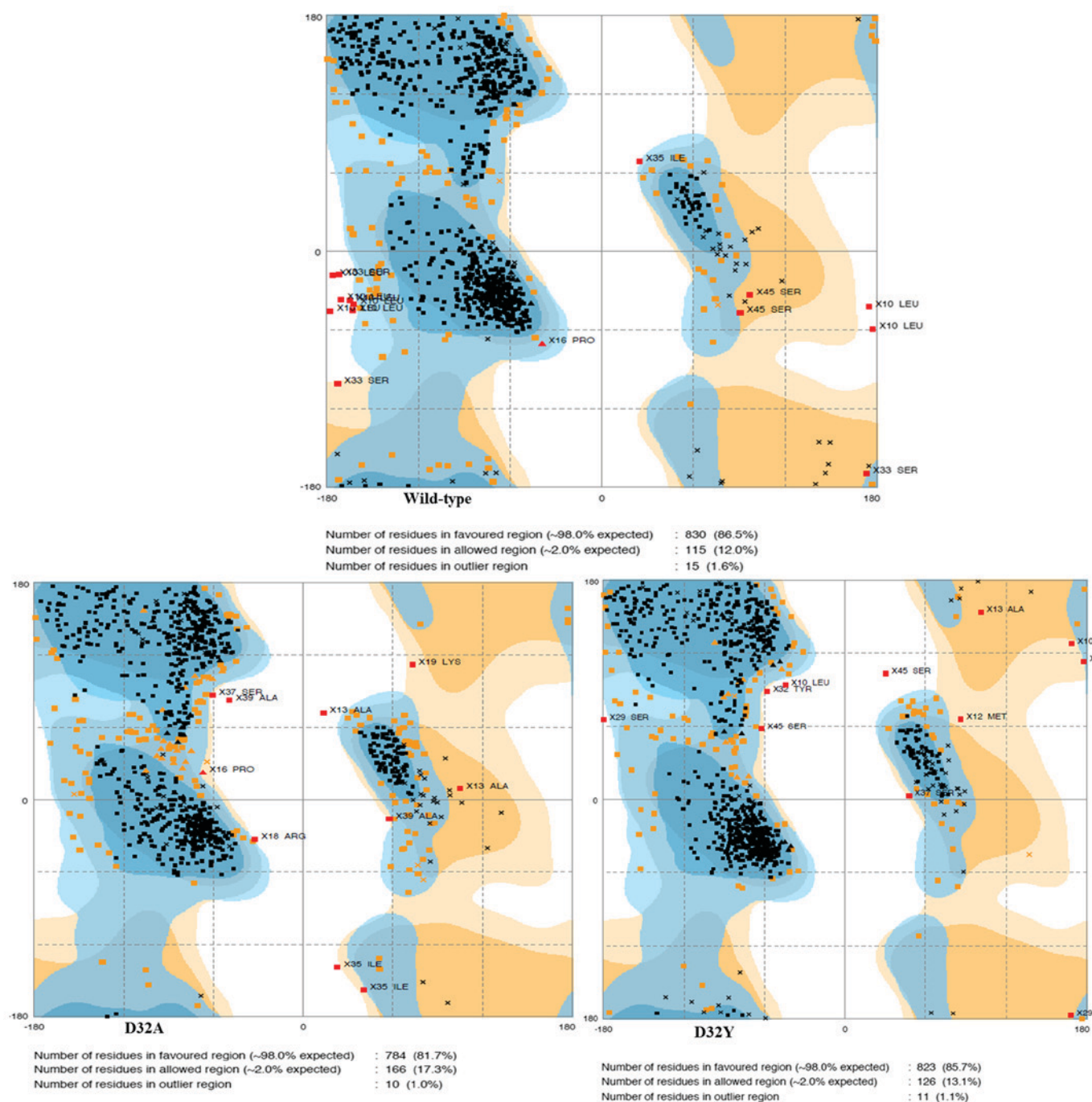


Figure 3. Ramachandran plot of  $\beta$ -catenin structure from the molecular dynamics simulation. A plot was obtained from the average of 20 snapshots from the 100-200-nsec trajectory. In all cases, <1% of total amino acids fell within the outlier (disallowed) region. These data indicate the validity of simulated structure throughout the simulation.

coil in the fragment structure. The plot illustrated that <1% of the overall amino acids in the dynamics trajectories fell into the outlier (disallowed) region (Fig. 3). Initially, the P16-H28 fragment was predicted to be a helical structure in the first place (Fig. 2). In the wild-type protein, after 100 nsec, the secondary structure of P16-H28 remained helical, similar to the starting structure (Fig. 4). These results indicated that the helical structure of this protein fragment (P16-H28) is a prerequisite to S33 phosphorylation. A similar pattern of secondary structure was observed in the D32Y fragment (Fig. 5). This indicated that D32Y mutation, at least, may not affect phosphorylation activity via conformation distortion in

this area. However, in the D32A mutant, a loss in the helical secondary structure of P16-H28 was observed, moving from a helical shape into a turn (Fig. 6). The D32A mutant therefore clearly possesses structural differences to the wild type  $\beta$ -catenin, and the mutation could enhance protein unfolding via alternation of the helical structure to interfere with the functional S33 position.

## Discussion

SPN belongs to a group of human cancer types that occur in developing organs with somatic mutations to *CTNNB1*.

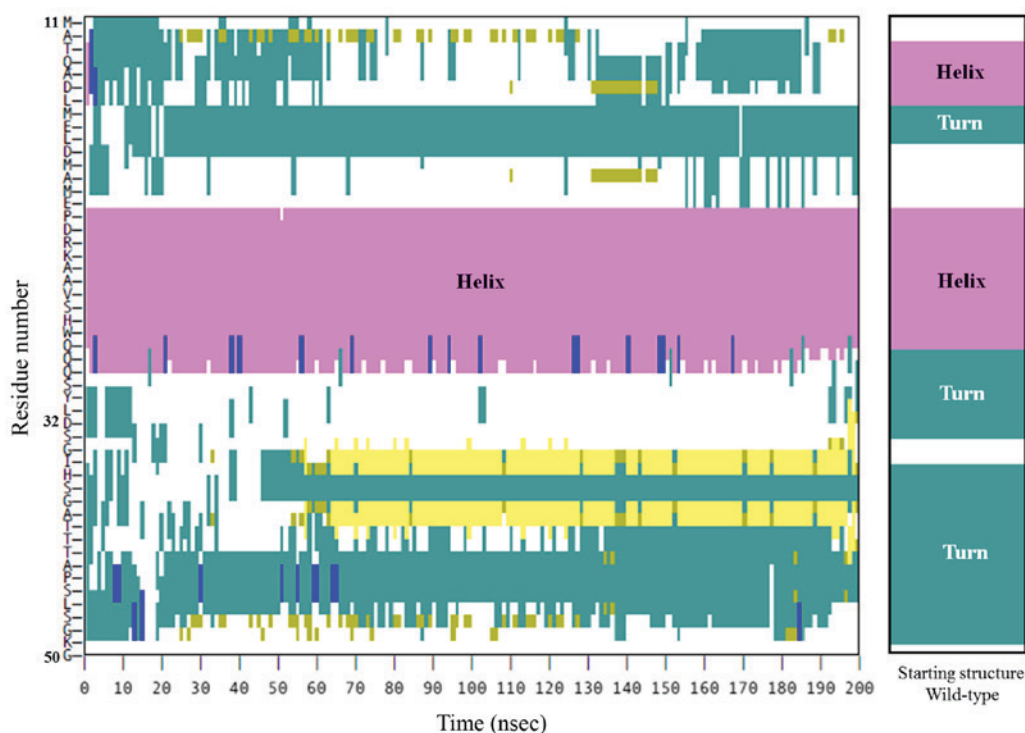


Figure 4. Secondary structure of wild-type  $\beta$ -catenin from molecular dynamics simulation, plotted against the simulation time. The number 32 indicates the position of the amino acid D32. The P16-H28 region remained helical throughout the simulation.

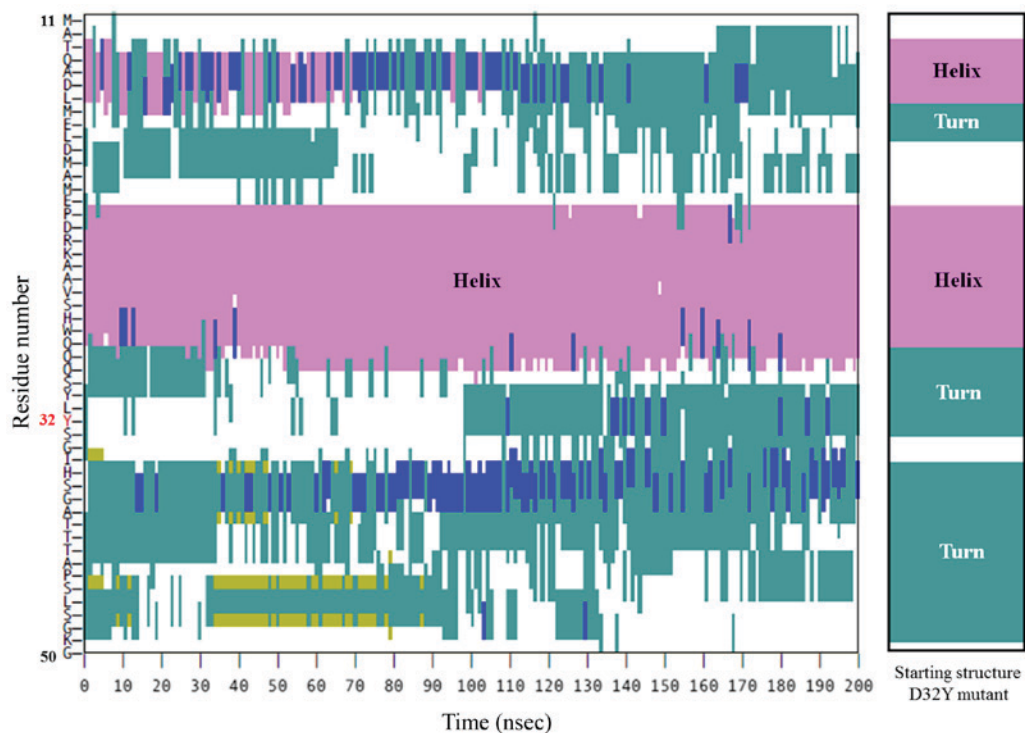


Figure 5. Secondary structure of D32Y  $\beta$ -catenin from molecular dynamics simulation, plotted against the simulation time. The number 32 indicates the position of the mutated amino acid Y32. The P16-H28 region remained helical throughout the simulation, similar to the wild-type structure.

Patterns of *CTNNB1* mutation differ, and are specific to tumor types. In nephroblastoma, *CTNNB1* mutations usually occur to codon 45, whereas the majority of mutations in hepatoblastoma are large deletions involving exon 3 (10,35). Defective phosphorylation caused by  $\beta$ -catenin

sequence alterations involves the priming phosphorylation sites for casein kinase I proteins, underlying the molecular mechanism of tumorigenesis of those neoplasms. Tumors containing mutations on the main phosphorylation sites are relatively fast-growing, invasive and respond well to

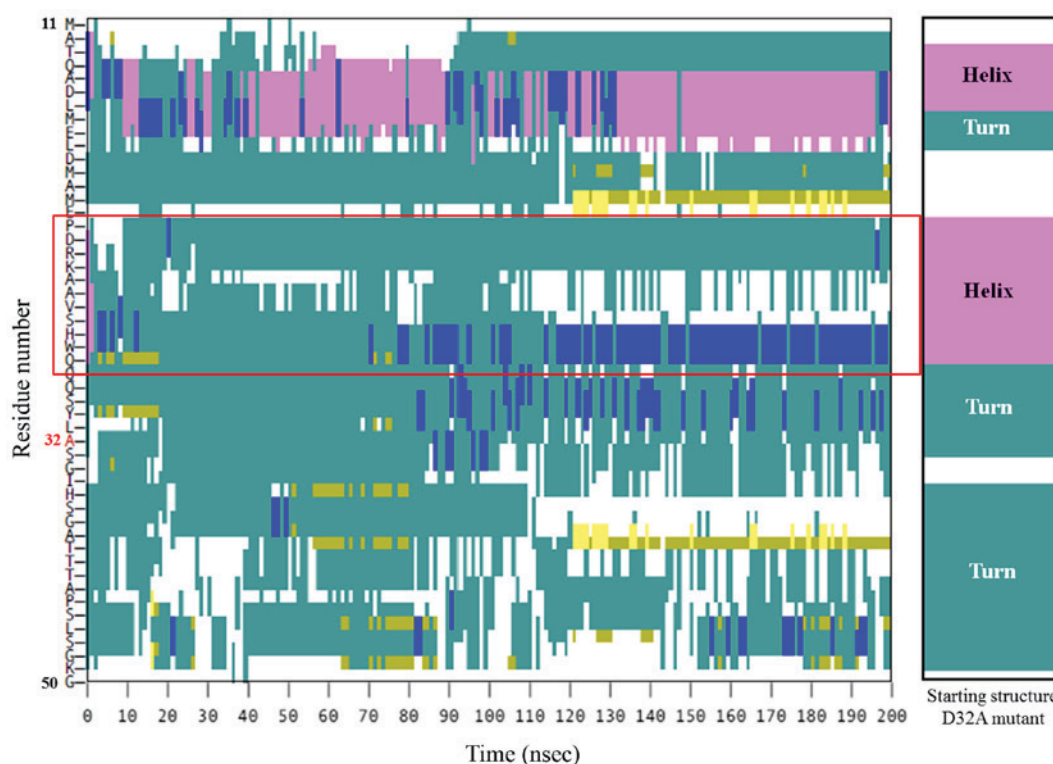


Figure 6. Secondary structure of D32A  $\beta$ -catenin from molecular dynamics simulation, plotted against the simulation time. The number 32 indicates the position of the mutated amino acid A32. The P16-H28 region, depicted with a red rectangular, lost its helical structure and became a turn following a simulation time of <10 nsec. This result indicated D32A could induce a conformational change in  $\beta$ -catenin and lead to interference of phosphorylation.

chemotherapy. The mutation spots in medulloblastomas and pancreaticoblastomas are confined to residues 33 and 37, which are sequential phosphorylation sites for GSK-3 $\beta$  (10). Tumors harboring lesions on those secondary phosphorylation sites are usually found in older children and are relatively non-invasive (10).

Alterations to *CTNNT1* codon 32 have been reported in rare tumor types, including SPN, pilomatrixomas and medulloblastomas (36-38). These tumors are relatively low-grade and rarely undergo distant metastasis. The study of Ellison *et al* (36) demonstrated that codon 32 was the most commonly mutated in childhood medulloblastoma. The current study detected mutations to this codon in each of the three cases studied. Together, this evidence supports the relevance of the molecular pathology in these rare tumors.

Three-dimensional molecular simulation is a useful computational tool for the prediction of the molecular structure of biomolecules, particularly proteins. The present study demonstrated that amino acid alterations to codon 32 tend to interfere with a helical structure within  $\beta$ -catenin. The MD simulations indicated that the D32A mutation was responsible for hindrance of phosphorylation at S33 in  $\beta$ -catenin by contributing to a loss of secondary structure, although D32Y may not act in the same way. Data from the structural prediction were consistent with a previous functional genetic study by Al-Fageeh *et al* (13), which demonstrated increased T-cell factor transactivation in a 293 cell culture model.

In conclusion, the present study used a computer-generated molecular structure model to successfully predicted conformational changes to  $\beta$ -catenin caused by point mutations at codon 32. These data indicate at the mechanism of

tumorigenesis in patients with SPN that possess D32  $\beta$ -catenin mutations.

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#### Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author, on reasonable request.

#### Authors' contributions

VT and NCP performed *in silico* molecular modeling of the  $\beta$ -catenin protein. KK interpreted the pathological and immunohistochemistry results. JS performed the mutation study and wrote the manuscript. SS collected the clinical data.

#### Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of the Faculty of Medicine, Prince of Songkla



University (Hat Yai, Thailand) and all patients provided written informed consent.

### Consent for publication

Patients provided written informed consent for the publication of their data.

### Competing interests

The authors declare that they have no competing interests.

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