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β-Catenin alterations in testicular Leydig cell tumour: a immunohistochemical and molecular analysis

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β-Catenin alterations in testicular Leydig cell tumour: a immunohistochemical and molecular analysis

Background: Testicular Leydig cell tumours (LCTs) are the most common type of sex cord–stromal tumour in men, representing 1%–3% of all testicular neoplasms. Among testicular sex cord–stromal tumours, *CTNNB1* mutations and nuclear expression of β-catenin have been typically associated with Sertoli cell tumour. Recent genomic analyses have shown that *CTNNB1* variants are also identified in a subset of LCTs; however, the frequency and clinicopathologic associations of β-catenin alterations remain incompletely understood in this tumour type.
Methods: In this study, we evaluated 32 LCTs (five malignant/metastasizing, 27 nonmetastasizing) using β-catenin immunohistochemistry and DNA sequencing.
Results: Immunohistochemistry revealed focal or multifocal nuclear β-catenin expression in 47% of the tumours. Diffuse nuclear β-catenin expression (in >50% of the tumour cells) was not detected in any of the cases analysed herein. Comparison of β-catenin--positive and β-catenin-negative cases did not show significant differences in the frequency of adverse histopathologic findings or malignant clinical behaviour. DNA sequencing performed *de novo* on a subset of seven cases revealed the presence of exon 3 *CTNNB1* variants in four of them (4/7, 57%), with variant allele frequencies (VAF) ranging from 7 to 33%. Two additional β-catenin-positive cases that had been sequenced as part of a previous study harboured exon 3 *CTNNB1* variants at VAF of 28% and 7%, respectively.
Conclusion: These results demonstrate that β-catenin alterations are relatively common in LCT, most likely occurring as subclonal events that are not enriched in cases with aggressive features. Further studies are needed to clarify the oncogenic role of β-catenin in this tumour type.

Keywords: *CTNNB1*, Leydig cell tumour, sex cord-stromal tumour, testis, β-catenin

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Introduction

In the testis, Leydig cell tumours (LCTs) are the most common sex cord–stromal tumour, accounting for ~3%–5% of all testicular neoplasms.[1] Their incidence is bimodal, with peaks in prepubertal children and in adults 30–60 years of age, respectively.[1,2] A small subset of LCTs (~10%) are clinically malignant, and these occur exclusively in postpubertal patients.[3] Although malignant LCTs usually exhibit aggressive histopathologic features, proper risk assessment often requires a combined evaluation of clinical, morphologic, and molecular data.[2–5]

Among testicular sex cord–stromal tumours, pathogenic *CTNNB1* variants and diffuse nuclear expression of β-catenin are characteristic of many Sertoli cell tumours, with nuclear β-catenin positivity reported in ~70% of cases overall.[6,7] In a recent study, gain-of-function *CTNNB1* variants or inactivating *APC* variants were identified in most (~90%) benign and in half of malignant Sertoli cell tumours.[8] Genomic analyses of a limited number of LCTs have demonstrated that pathogenic *CTNNB1* variants are also present in a subset of these neoplasms, including cases with aggressive histopathologic features and malignant clinical behaviour.[5] However, the frequency, patterns, and clinicopathologic correlates of β-catenin alterations in LCT remain incompletely described. In this study, we sought to assess the prevalence of nuclear β-catenin expression in LCTs and explore its clinicopathologic and genomic correlates.

Material and Methods

IDENTIFICATION OF CASES AND PROCUREMENT OF TISSUE

This research was approved by the Institutional Review Board of the Indiana University School of Medicine (IRB: #18697, 2023) and the remaining participating institutions (when applicable). Institutional pathology archives (Indiana University, Indianapolis, IN, USA), personal consultation files (TMU), and research files (AMA) were queried to identify cases diagnosed as LCTs in the testis or in metastatic sites (in male patients). Available haematoxylin and eosin-stained (H&E) slides were retrieved and centrally reviewed (Y.K. and A.M.A.) to confirm the diagnosis; cases with archival, formalin-fixed, paraffin-embedded tissue were further selected for inclusion.

ASSESSMENT OF CLINICOPATHOLOGIC FEATURES AND IMMUNOHISTOCHEMISTRY (IHC)

Electronic medical records, pathology reports, and available H&E slides were reviewed to collect relevant clinical and pathologic data. The following parameters were recorded for each case: patient age (dichotomized as <40 versus ≥40 years), type of tumour (nonmetastasizing versus metastasizing), tumour size (dichotomized as ≤5 cm versus >5 cm), mitotic activity (dichotomized as ≤3 versus >3 per 10 high-power fields), marked nuclear atypia/pleomorphism, tumour necrosis, lymphovascular invasion, and destructive/invasive growth. Metastasizing LCTs were defined as tumours with biopsy-proven metastases.

Immunohistochemistry was performed on a single representative section per case with a monoclonal anti-β-catenin antibody (Clone 14, Cell Marque, Rocklin, CA, USA; RTU). Antigen retrieval was performed using a low-pH method, and the signal was generated using the Envision Plus DAB detection system (Dako, Carpinteria, CA, USA).[7] Immunohistochemistry was interpreted as positive when there was at least focal nuclear β-catenin expression; tumours showing only membranous and/or cytoplasmic staining were considered negative. In positive cases, β-catenin expression was qualitatively interpreted as focal/multifocal if there was one or more discrete (i.e. nonconfluent) foci with nuclear reactivity or diffuse if there were extensive confluent areas of nuclear reactivity comprising more than half of the tumour present in the tissue section. Positive staining was further assessed semi-quantitively as <10%, 10%–25%, >25%–50%, and >50%, based on the fraction of tumour cells exhibiting nuclear β-catenin expression.

DNA SEQUENCING

DNA sequencing was performed at the University of Bologna using a laboratory-developed panel that analyzes selected genomic regions of 28 genes (*BRAF*, *CTNNB1*, *DICER*, *DPYD*, *EGFR*, *EIF1AX*, *GNA11*, *GNAQ*, *GNAS*, *H3F3A*, *HRAS*, *IDH1*, *IDH2*, *KIT*, *KRAS*, *MED12*, *MET*, *NRAS*, *PDGFRα*, *PIK3CA*, *PTEN*, *RET*, *RNF43*, *SMAD4*, *TERT*, *TP53*, *TSHR*, and *VHL*; human reference sequence hg19/GRCh37).[9] The specific regions of *CTNNB1* covered by this assay include exons 3, 7, and 8. Briefly, ~30 ng of DNA was used to construct sequencing libraries using a commercial kit according to the manufacturer's recommendations (AmpliSeq Plus Library Kit 2.0, Thermo Fisher Scientific, Waltham, MA, USA).

Sequencing was performed on an Ion S5 Prime machine and the results were analysed with the Ion Reporter tools (v5.18—Thermo Fisher Scientific). As described in the validation study of the assay,[10] single nucleotide variants/indels present in at least 5% of the generated reads and observed in both strands were considered for variant calling. The Varsome tool (https://varsome.com/)[9] was used to evaluate the ACMG (American College of Medical Genetics and Genomics) classification, AMP score, and ClinVar classification of each reported variant. Sequencing results were further assessed for clinical and biologic relevance by one of the authors with expertise in molecular pathology (DdB).

STATISTICS

Differences between groups were assessed using Fisher's exact test (R Project for Statistical Computing, The R Foundation, Vienna, Austria). A *P*-value of <0.05 was considered the threshold for statistical significance.

Results

Thirty-two (32) LCTs were evaluated, including five metastasizing and 27 nonmetastasizing tumours. β-catenin was positive in 15/32 (47%; one metastasizing and 14 nonmetastasizing) tumours and negative in 17/32 (53%, four metastasizing and 13 nonmetastasizing) LCTs. In all cases, nuclear β-catenin expression was focal or multifocal, with no case demonstrating diffuse nuclear expression (Figures 1 and 2). More specifically, β-catenin expression was present in <10% of lesional cells in 9/15 tumours (60%), 10%–25% of lesional cells in 4/15 tumours (27%), and >25%–50% of lesional cells in 2/15 tumours (13%).

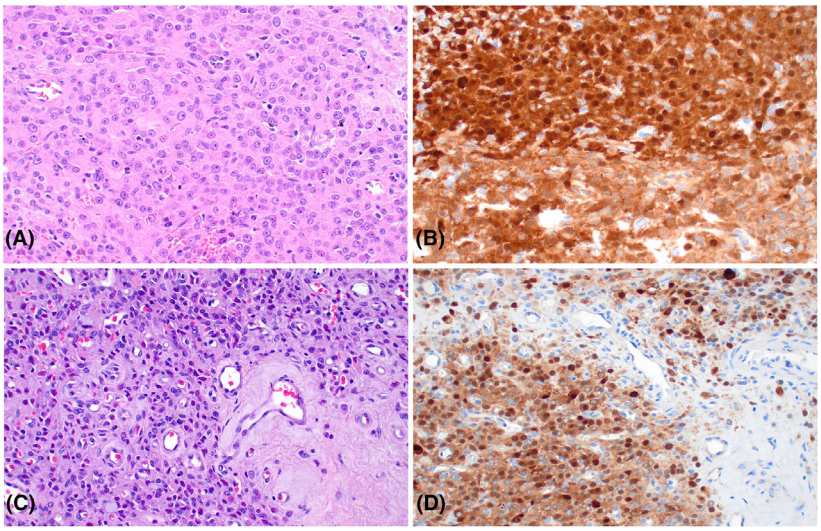

Figure 1. Benign (nonmetastasizing) Leydig cell tumours with β-catenin expression. **A**: This tumour was sequenced as part of a prior study and harboured *CTNNB1* p.G34R at a variant allele frequency of 28%. **B**: Immunohistochemistry demonstrated nuclear β-catenin expression in >25%–50% of the cells. **C**: This tumour was sequenced *de novo* and harboured *CTNNB1* p.H36_S37del at a variant allele frequency of 33%. **D**: Immunohistochemistry demonstrated nuclear β-catenin expression in 10%–25% of the cells.

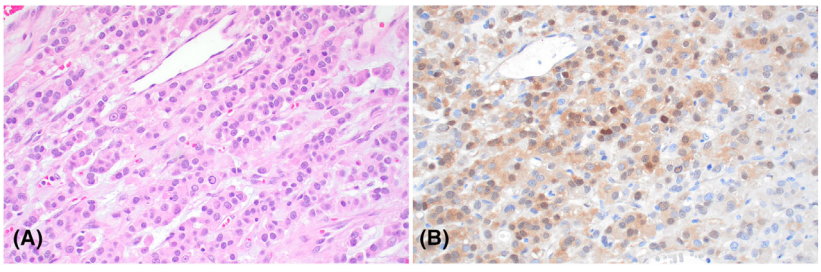

Figure 2. Malignant (metastasizing) Leydig cell tumour with β-catenin expression. **A,B**: This was the only metastasizing with nuclear β-catenin expression (<10% of the tumour cell nuclei).

Comparison of β-catenin-positive and β-catenin-negative cases showed no significant differences in patient age, tumour size, mitotic activity, and frequency of nuclear pleomorphism, invasive growth, lymphovascular invasion, tumour necrosis, and clinical behaviour between positive and negative cases (Table 1).

Table 1. Clinicopathologic features of Leydig cell tumours with and without beta-catenin expression

Clinicopathologic features	β-Catenin −	β-Catenin +	*P* for difference
Age			
>40	6	5	0.2
≤40	11	7	
NA	0	3	
Classification			
Nonmetastasizing	13	14	0.3
Metastasizing	4	1	
Size*			
≥5 cm	1	1	0.7
<5	12	11	
NA	0	2	
Mitoses*			
≤3	12	13	1
>3	1	1	
Atypia*			
Absent	9	11	0.7
Present	4	3	
Invasive growth*			
Absent	11	11	1
Present	2	2	
NA	0	1	
LVI*			
Absent	12	14	0.5
Present	1	0	
Necrosis*			
Absent	12	14	0.5
Present	1	0	

*Features assessed only in nonmetastasizing cases.

DNA sequencing was performed on a subset of seven LCTs with additional formalin-fixed paraffin-embedded material available to assess whether nuclear β-catenin expression correlated with underlying pathogenic *CTNNB1* variants. Two additional β-catenin-positive LCTs had been previously sequenced by our group in a prior study.[5] Sequencing demonstrated that 4/7 (57%) of the LCTs analysed *de novo* harboured exon 3 *CTNNB1* variants expected to be pathogenic. All these variants involved a region of the N-terminal domain targeted by kinases that mark β-catenin for recognition by ubiquitin ligases and subsequent proteasomal degradation. The specific variants identified included *CTNNB1* p.S33Y (variant allele frequency [VAF] 26%), *CTNNB1* p.T41I (VAF 30%), *CTNNB1* p.D32V (VAF 9%), and *CTNNB1* p.H36_S37del (VAF 33%). In the remaining three cases sequenced *de novo*, no pathogenic *CTNNB1* variants were identified. Of note, the LCT with *CTNNB1* p.D32V (VAF 9%) also harboured a *RET* variant (p.R844L, VAF 33%). This change results in the replacement of a charged amino acid residue (R) for an uncharged aliphatic amino acid residue (L) in the tyrosine kinase domain of the protein and is expected to have functional impact. However, this variant has not been previously annotated in COSMIC and it has been reported as a germline event[11]; therefore, its pathogenic role in this context remains uncertain. The two LCTs sequenced as part of a prior study harboured *CTNNB1* p.G34R (VAF 28%) and *CTNNB1* p.H36_T42del (VAF 7%).[5] Pairwise comparisons between the VAFs of the *CTNNB1* variants and the percentage of cells with nuclear β-catenin expression in the seven cases with positive sequencing results were: IHC <10%/VAF 9%, IHC 10%–25%/VAF 33%, IHC10-25%/VAF 30%, IHC10-25%/VAF 26%, IHC 25%–50%/VAF 7%, and IHC 25–50%/VAF 28%. In the three cases without *CTNNB1* variants, the percentage of cells with nuclear β-catenin expression was <10%, <10%, and 10%–25%, respectively.

Discussion

Among testicular sex cord–stromal tumours, pathogenic *CTNNB1* variants resulting in nuclear β-catenin expression are characteristically associated with Sertoli cell tumour.[6,7,12] In this tumour type, gain-of-function *CTNNB1* variants likely represent the main oncogenic driver and correlate with diffuse nuclear expression of β-catenin (Figure 3). Recently, pathogenic *CTNNB1* variants have also been identified in

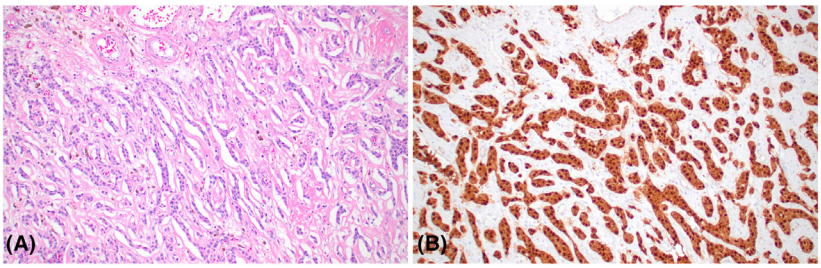

Figure 3. Nuclear β-catenin expression in Sertoli cell tumour. **A,B**: Unlike Leydig cell tumour, Sertoli cell tumour typically demonstrates diffuse nuclear β-catenin expression in >95% of tumour cells.

LCTs, possibly as subclonal (rather than main driver) alterations.[5] In this context, subclonal *CTNNB1* mutations may represent stochastic (i.e. random) passenger events without major biologic significance or, alternatively, they may confer an evolutionary advantage and promote progression to a more aggressive neoplastic phenotype. However, the clinicopathologic and genomic correlates of nuclear β-catenin expression remain incompletely described.

The present study demonstrates that a significant proportion of LCTs (~50%) express nuclear β-catenin, and this correlates with the presence of underlying pathogenic *CTNNB1* variants in most of them. In this study 3/7 LCTs (43%) sequenced *de novo* (3/9, 33% of all cases sequenced) did not harbour pathogenic *CTNNB1* variants. In these tumors, the presence of nuclear β-catenin expression may correlate with variants in regions of the gene that were not assessed by the panel. Alternatively, β-catenin may be functionally upregulated as a consequence of other events, including alterations in genes that are key regulators of the protein (such as *APC*) but are not covered by the assay.[5,8,13] Moreover, the possibility that nuclear β-catenin represents a "false positive" result in a subset of tumours cannot be entirely excluded, especially when positivity for this marker is limited (<10%).

As suggested in a prior study,[5] the pattern of nuclear β-catenin expression seen in the LCTs analysed in this series is consistent with subclonal activation of the protein (and *Wnt* signalling). Of note, the frequency of nuclear β-catenin expression did not differ between cases with and without malignant clinical behaviour and/or adverse histopathologic findings, suggesting that β-catenin alterations may represent "passenger" events. However, their high frequency (~50%) suggest that they are not random/stochastic alterations in this tumour type. It is possible that, for unknown reasons, LCTs are predisposed to acquire *CTNNB1* alterations that do not confer a subclonal evolutionary advantage; however, further studies are needed to explore this hypothesis.

In conclusion, our study demonstrates that nuclear β-catenin expression and *CTNNB1* variants are highly prevalent in LCTs. The subclonal nature of these variants, which correlates with focal or multifocal expression of nuclear β-catenin demonstrated by IHC, can be used to distinguish LCTs from Sertoli cell tumour (Figure 3). The results presented herein suggest that β-catenin alterations and gain-of-function *CTNNB1* variants are not associated with aggressive behaviour in LCT, although definitive conclusions in this regard are limited by the small number of malignant cases included in the series. Additional studies are needed to clarify any possible oncogenic role of β-catenin alterations in this tumour type.

Author contributions

Andres M. Acosta: Concept, design, and coordination; Thomas M. Ulbright, Andres M. Acosta, Kristine M. Cornejo, Costantino Ricci: Material; Dario De Biase: DNA sequencing; Yukiko Kitagawa and Andres M. Acosta: Data analysis and interpretation; Yukiko Kitagawa and Andres M. Acosta: Article draft. All authors were involved in the article editing and intellectual contributions.

Conflict of interest statement

The authors declare that they have no financial or intellectual conflicts of interest pertaining to the contents of this article.

Data availability statement

The data generated for this research are available from the corresponding author upon reasonable request.

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