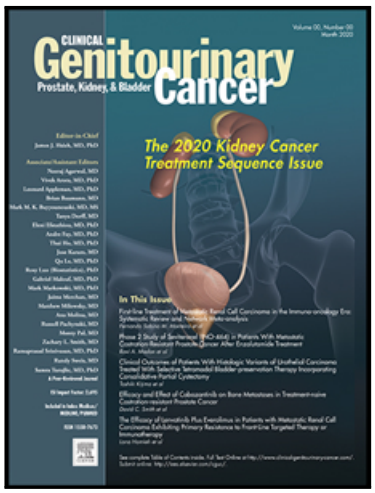

TERT gene fusions characterize a subset of metastatic Leydig cell tumors

Bozo Kruslin , Zoran Gatalica , Ondrej Hes , Faruk Skenderi , Markku Miettinen , Elma Contreras , Joanne Xiu , Michelle Elis , Elena Florento , Semir Vranic , Jeffrey Swensen

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Highlights

- Metastatic Leydig cell tumors are rare and difficult to treat malignancies without known underlying molecular-genetic events.
- We identified *TERT* gene fusions exclusively in malignant, metastatic Leydig cell tumors.
- Additional predictive biomarkers (TOP1 and AR) may help guide decisions on chemo- and/or hormone therapy for selected individual patients.

TERT gene fusions characterize a subset of metastatic Leydig cell tumors

Running title:

Profiling of Leydig cell tumors

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MicroAbstract

Metastatic Leydig cell tumors (LCT) are rare, difficult to treat malignancies without known underlying molecular-genetic events. We profiled 27 LCT cases using NGS and immunohistochemistry. Our study identified *TERT* gene fusions as a main genetic alteration and a potential therapeutic target in LCT. TOP1 and AR expressions may guide decisions on chemo- and/or hormone therapy for selected individual patients.

CRediT author statement

Bozo Kruslin: Formal analysis, resources, writing – original draft preparation

Zoran Gatalica: Conceptualization, data analysis, writing – original draft preparation, supervision

Ondrej Hes: Formal analysis, resources

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Elma Contreras: Formal analysis, validation

Joanne Xiu: Formal analysis, validation

Michelle Elis: Formal analysis, validation

Elena Florento: Formal analysis, validation

Semir Vranic: data analysis, writing – original draft preparation, supervision

Jeffrey Swensen: Conceptualization, data analysis, writing – original draft preparation, supervision

Acknowledgement

The preliminary results from the current study were presented at the 44th European Society of Medical Oncology Congress (ESMO 2019) that was held in period 27 September through 1 October 2019 in Barcelona, Spain.

Conflict of Interest

Elma Contreras, Joanne Xiu, Elena Florento, Michelle Elis and Jeffrey Swensen are employees of Caris Life Sciences. Zoran Gatalica reports Caris stock ownership. Other authors declare no conflict of interest.

Abstract

Objective: Metastatic Leydig cell tumors (LCT) are rare, difficult to treat malignancies without known underlying molecular-genetic events. An index case of metastatic LCT showed an *LDLR-TERT* gene fusion upon routine genetic profiling for detection of therapeutic targets, which was then followed by an investigation into a cohort of additional LCTs.

Patients and Methods: Twenty-nine LCT (27 male and 2 female patients) were profiled using NGS and immunohistochemistry.

Results: *TERT* gene fusions were detected only in testicular metastatic Leydig cell tumors, in three of seven successfully analyzed cases (*RMST:TERT*, *LDLR:TERT* and *B4GALT5:TERT*). *TOP1* and *CCND3* amplifications were identified in the case with a *B4GALT5:TERT* fusion. A *TP53* mutation was detected in one metastatic tumor without a *TERT* fusion. Five primary (four testicular and one ovarian) LCTs showed multiple gene amplifications, without a consistent pattern. A single metastatic ovarian LCT showed *BAP1* mutation and copy number amplifications affecting the *NPM1, PCM1* and *SS18* genes. At the protein level, 4/7 metastatic and 6/10 primary testicular LCTs over-expressed TOP1. Androgen receptor (AR) was overexpressed in 10/13 primary testicular tumors and 2/5 metastatic testicular LCT (without detectable ARv7 mRNA or ARv7 protein). Only one metastatic testicular LCT exhibited high TMB while all tested cases were MSI stable and did not express PD-L1.

Conclusions: Our study for the first time identified *TERT* gene fusions as a main genetic alteration and a potential therapeutic target in metastatic Leydig cell tumors. TOP1 and AR may guide decisions on chemo- and/or hormone therapy for selected individual patients.

Keywords: Sex cord–stromal tumors, Leydig cell tumor, molecular profiling, sequencing, targeted therapy.

Introduction

Sex cord–stromal tumors are an uncommon group of neoplasms affecting gonads. In testis, these tumors represent 4% of all neoplasms and are the second largest group of primary tumors after germ cell tumors [1]. In ovary, sex cord-stromal tumors constitute 5% of all neoplasms while 7% of malignant ovarian neoplasms belong to this group [2]. Leydig cell tumors (LCT) are the most common pure form of sex cord-stromal tumor followed by Sertoli cell, granulosa cell, and pure stromal tumors [1]. Little is known about the pathogenesis of these neoplasms beyond their rare association with germline fumarate hydratase (*FH*) mutations [hereditary leiomyomatosis and renal cell carcinoma syndrome (HLRCC), OMIM#150800] or the activating mutations that affect luteinizing hormone receptor (*LHR*) in the pediatric population [1]. In addition, *DICER1* mutations have been reported in sporadic and hereditary ovarian sex cord stromal tumors [3-6]. *DICER1* gene mutations have been implicated in the dysregulation of the steroid hormone synthesis including androgen (AR)[7].

Molecular profiling studies on these tumors are sparse due to the overwhelmingly benign course of the disease and curative surgical resection [8]. A recent whole exome sequencing study of Yuan et al. revealed that LCTs frequently harbor somatic mutations of *CDC27* (53%), *DICER1* (21%), and *MUC22* (21%) genes [8]. Metastatic LCTs are clinically challenging and without a consensus treatment approach.

We have previously characterized multiple cancers using comprehensive molecular profiling approach that utilizes various molecular techniques for the identification of potentially targetable biomarkers[9-13]. Our initial case of metastatic LCT showed an *LDLR:TERT* gene fusion

upon routine genetic profiling for detection of therapeutic targets. This led us to investigate a cohort of additional LCTs.

Materials and Methods

Samples for the study

Twenty-nine LCTs from five participating institutions (listed in affiliations#1, 3, 4, 5, 7 and 7) were included in the current study.

Prior to molecular testing, each LCT case underwent confirmation of the histological diagnosis and a review of the diagnostic immunohistochemical work-up performed at the referring/participating pathology laboratories. For the study, all histopathological reports and remnant LCT tissue samples provided by the referring laboratories and participating institutions were de-identified. Based on this, the study was compliant with 45 CFR 46.101(b) and was deemed exempt from Institutional Review Board (IRB) approval and consent requirements were waived.

All molecular assays were performed at a CLIA/CAP/ISO15189/NYSDOH certified clinical laboratory (Caris Life Sciences, Phoenix, AZ).

Immunohistochemistry (IHC)

PD-L1 expression was assessed in the tumor (TC) and immune cells (IC) using SP142 antibody (Ventana). PD-L1 expression was considered positive if either TC or IC exhibited any membranous/cytoplasmic staining [11, 14]. Androgen receptor (AR; clone 441, Leica Biosystems, Buffalo Grove, IL) was analyzed using a ≥10% threshold for nuclear positivity [12-14]. The ARv7 splice variant was explored at the protein level by IHC (EPR15656; Abcam) and at the mRNA level using anchored multiplex PCR for targeted RNA sequencing (ArcherDX) [9, 11].

Topoisomerase 1 (TOP1) expression (clone 1D6, Leica Biosystems, Germany) was scored as 0+, 1+, 2+, or 3+ depending on the staining intensity, and the percent tumor stained was also recorded. The threshold for TOPO1 overexpression was a staining intensity of ≥2+ in ≥30% of cancer cells [15].

Next-generation sequencing (NGS)

The LCT samples were profiled using next-generation sequencing (NGS) of exons from 592 genes (SureSelect XT, Agilent, Santa Clara, CA and the NextSeq instrument, Illumina, San Diego, CA). A full gene panel is available in the Supplemental Table 1.

The tumor mutational burden (TMB) was assessed by calculating the number of non-synonymous missense mutations, excluding common germline variants, per one megabase of DNA. TMB was considered high if ≥10 mutations/megabase (muts/Mb) were detected [16].

Microsatellite instability (MSI) was calculated from the NGS data by direct analysis of short tandem repeat tracts in the target regions of sequenced genes. The count only included alterations that resulted in increases or decreases in the number of repeats; high microsatellite instability (MSI-H) was defined as ≥46 altered microsatellite loci. This threshold was established by comparing NGS with the PCR-based microsatellite fragments analysis results from ~2100 samples [10].

Copy number amplifications (CNAs) were assessed by comparing the depth of detected NGS sequence reads to calibrated control values. Genes having ≥ six copies were considered amplified.

The ArcherDx FusionPlex Assay (ArcherDX, Boulder, CO) was used for gene fusion assessment. The gene fusions panel (n=54) is available in the Supplemental Table 2.

Results

Clinicopathological characteristics of the cohort

Twenty-seven testicular (Seven metastatic and twenty primary tumors) and two ovarian LCT (One metastatic and one primary) were investigated. The mean age was 55.5 years (range, 23-94 years) for male patients; the two female patients with ovarian LCT were 45 and 69 years, respectively. The metastatic sites of testicular LCT included lung, liver, mediastinum, parasternal region and retroperitoneum (x3). The only ovarian metastatic LCT site was peritoneum (four years following the original ovarian tumor diagnosis).

Immunohistochemical biomarkers

Topoisomerase 1 (TOP1) was assessed in 17 testicular LCT: 6 of 10 primary (60%) and four of 7 metastatic (57%) LCT were positive (Table 1 and Figure 1). Intriguingly, a single *TOP1* amplified testicular LCT showed no TOP1 protein expression by IHC. AR expression was more prevalent among the primary testicular LCT (10/13) compared with the metastatic cases (2/5). All cases were ARv7 negative (at either mRNA or protein levels).

Genomic characteristics of LCT

TERT (Telomerase Reverse Transcriptase) gene fusions were exclusively seen in three of seven successfully analyzed metastatic testicular LCT. The following fusions were detected: *RMST:TERT*, *LDLR:TERT* and *B4GALT5:TERT* (Figure 2). The specimen harboring the *B4GALT5:TERT* fusion also showed amplifications (>6 copies) of the *TOP1* and *CCND3* genes (Table 1). Neither of the two ovarian LCT harbored *TERT* related fusions. *TERT* promoter

mutations were not tested, because this region was not covered in the available commercial NGS panel at the time.

A next generation sequencing mutational profile was available for 15 testicular cases, which showed inconsistent and rare pathogenic mutations: two primary LCT harbored *CTNNB1* gene mutations (encoding beta-catenin protein); *FOXO4* mutations were also observed in two cases (one primary and one metastatic case) while a *TP53* mutation was observed in one metastatic LCT. All other mutations were detected in single cases (*NBN, MTOR, BAP1, MEN1,* and *CREBBP*) (Table 1). A single metastatic ovarian LCT had a *BAP1* mutation and copy number amplifications of the *NPM1, PCM1* and *SS18* genes.

Copy number amplifications were detected in 8 out of 18 successfully tested cases (6 testicular and two ovarian LCTs). The more prevalent CNAs included those affecting *CCND3* (two testicular) and genes in the fibroblast growth factor family: *FGF3* (one primary ovarian), *FGFR3* (one primary testicular and one primary ovarian) and *FGFR4* (one metastatic testicular) (Table 1).

Immuno-Oncology (I-O) Biomarkers

PD-L1 expression (threshold ≥1%) in the TC or IC was not seen in any of 15 tested testicular LCTs. All cases were MSI stable. A low tumor mutation burden (4-7 muts/Mb) characterized most of the testicular LCT except the peculiar metastatic case with a *B4GALT5:TERT* fusion and *TOP1* and *CCND3* amplifications that exhibited 11 muts/Mb (Table 1).

Discussion

Our study represents the first comprehensive molecular study to examine potentially targetable molecular alterations in LCT including its malignant variants. One of the key findings in our study was that *TERT* gene fusions were a major detected genetic alteration in malignant, metastatic Leydig cell tumors. This is a novel finding that had not been previously reported in sex-cord stromal tumors including LCT [3, 5, 8]. In addition, all three described gene fusions affecting *TERT* gene have not been previously reported in the literature (review of the literature covered PubMed/MEDLINE and COSMIC database). TERT activity plays a central role in the unlimited self-renewal potential of cancer cells via telomerase activity that maintains telomere ends through addition of telomere repeats TTAGGG) [17]. This mechanism is considered one of the hallmarks of cancer [18]. Various genomic alterations including *TERT* promoter mutations, rearrangements, amplifications, fusions and promoter methylation have been well characterized across human cancers [19-21]. Limited information of the therapeutic implications of *TERT* genomic alterations are currently available. One recent in vitro study conducted on acral melanoma cells revealed the cytotoxic effects of TERT inhibitors in melanoma cells harboring *TERT* genomic alterations [21].

The family of topoisomerase enzymes (TOP1 and TOPO2) are the key players in unwinding coiled DNA to facilitate the cell replication and transcription [22]. Given their active role in DNA replication and transcription, several classes of drugs targeting TOP1 and TOPO2 have been developed. One of these drugs is camptothecin against TOP1 whose derivatives irinotecan and topotecan have been widely used as cytotoxic drugs in a clinical setting. TOP1 overexpression has been described in various cancers [15] whereas *TOP1* gene amplification is a

much rarer event in cancers [the highest amplification rate (>10%) was reported in gall bladder, esophageal and gastroesophageal carcinomas] [15]. Our study revealed a common (50-60%) TOP1 expression in both primary and metastatic LCT while *TOP1* gene amplification was observed in one metastatic case. This finding may be clinically relevant for malignant LCTs and provide a rationale for the treatment with camptothecin derivatives alone or combined with novel anticancer treatments such as antibody-drug conjugates (ADC) that contain irinotecan.

Hormone therapy with antiandrogens has been used therapeutically in prostate cancer patients [23]. Some of the commonly used antiandrogens (e.g. bicalutamide) competitively inhibit ligand binding to the active AR. Our study also confirmed AR activity in LCTs without the presence of splice variant ARv7. In prostate cancer cells, ARv7 stems from aberrant mRNA splicing of AR exons 1–3, loss of exons 4–8, and inclusion of cryptic exon 3 (CE3) into the transcribed *AR* gene [24, 25]. Consequently, the affected protein is constitutively active in the absence of androgens and facilitates the growth of prostate cancer in the presence of antiandrogens [26, 27]. We found AR expression in 40% of metastatic LCT without the ARv7 splice variant, which indicates a potential for treatment with antiandrogens.

Immunotherapy with immune checkpoint inhibitors against PD-1/PD-L1 has markedly improved the treatment and outcome of multiple solid and hematological cancers (e.g. non-small cell lung carcinoma, melanoma, renal cell carcinoma, urothelial bladder carcinoma, triple-negative breast carcinoma, classical Hodgkin lymphoma). Several currently available predictive biomarkers (PD-L1 expression, high TMB, MSI-H status) with approved clinical utility have been explored in this study. In contrast to testicular germ cell tumors [28, 29], we found no PD-L1

expression in LCTs. With the exception of one case with high TMB (11 muts/Mb), all cases exhibited a low TMB, and all cases were MSI stable. Based on these results, it is unlikely that these patients would benefit from targeted therapy from immune checkpoint inhibitors.

There are several limitations of our study. The lack of matched primary sample analysis for cases with *TERT* fusion-positive metastases to determine if the fusions represent early events in more aggressive cancers or later events associated with metastasis. If the fusions are early events, patients with fusion-positive primary tumors could have increased surveillance. In addition, the *TERT* promoter mutations, commonly observed in other malignancies (e.g., gliomas, bladder, thyroid cancers, melanoma), were not possible to examine in this study due to the lack of the gene promoter coverage in the NGS panel available at the time of study [30-33]. Finally, there is lack of feedback information on the usefulness of molecular profiling in the treatment of metastatic LCT with potentially actionable findings detected in our cohort (e.g., over-expression of TOP1 and AR).

In conclusion, we identified for the first time *TERT* gene fusions as a main genetic alteration and several potential therapeutic targets in malignant, metastatic Leydig cell tumorsincluding TOP1 and AR which may help guide decisions on chemo- and/or hormone therapy for selected individual patients.

CRediT author statement: JS and SV had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Bozo Kruslin: Formal analysis, resources, writing – original draft preparation

Zoran Gatalica: Conceptualization, data analysis, writing – original draft preparation, supervision

Ondrej Hes: Formal analysis, resources

Faruk Skenderi: Formal analysis, resources

Markku Miettinen: Formal analysis, resources

Elma Contreras: Formal analysis, validation

Joanne Xiu: Formal analysis, validation

Michelle Elis: Formal analysis, validation

Elena Florento: Formal analysis, validation

Semir Vranic: data analysis, writing – original draft preparation, supervision

Jeffrey Swensen: Conceptualization, data analysis, writing – original draft preparation, supervision

Data availability statement

The data presented in the current study are available from the corresponding authors upon reasonable requests.

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Tables

Biomarkers (number)	Testis (n=27)	
	Primary (n=20)	**Metastatic (n=7)**
Topo1α protein* (IHC) (n=17)	6/10 (60%)	4/7 (57%)
Androgen receptor* (AR) (n=18) ARv7 (NGS and IHC) (n=18)	10/13 (77%) All ARv7 negative (mRNA or protein)	2/5 (40%) All ARv7 negative (mRNA or protein)
	Genomic alterations	
TERT gene fusions (NGS)** (n=19)	**0/12 (0%)**	**3+/7 (43%):** *LDLR:TERT* *B4GALT5:TERT* *RMST:TERT*
Mutational profile (NGS) (n=15)	*CTNNB1* (2/10), *NBN* (1/10), *MTOR* (1/10), *FOXO4* (1/10), *BAP1* (1/10), *MEN1* (1/10), *CREBBP* (1/10)	*TP53* (1/5) *FOXO4* (1/5)
Copy number amplifications (NGS) (n=16)	*MDM2, TCF3, LRIG3, HMGA2, CYP2D6, ASPSCR1* (1 case) *CDKN1B, DAXX, DDX5, PER1, VEGFB* (1 case) *MDM2, CDK4, CCND3, TFEB* (1 case) *GATA3, FGFR3, AKT2, TLX1, PIK3R2, MEF2B, JAK3, ERCC2, ELL, CIC, CD79A, CBFA2T3, BCL3* (1 case)	*TOP1, CCND3, MCL1* (1 case) *FGFR4, FLT4* (1 case)
	I-O Biomarkers	
PD-L1 expression* (n=15)	0/10 (0%)	0/5 (0%)
Tumor mutational burden (TMB) (n=7)	4-7/Mb (n=4)	4-11/Mb (n=3)
Microsatellite instability (MSI) (n=7)	Stable (n=4)	Stable (n=3)
	Ovary (n=2)	
Biomarkers	**Primary (n=1)**	**Metastatic (n=1)**
Topo1α protein*	Not available	Not available
Androgen receptor (AR)*	Not available	Not available
	Genomic alterations	
TERT gene fusions (NGS)**	absent	absent
Mutational profile (NGS)	none	*BAP1*
Copy number amplifications (NGS)	*FGF3, FGFR3*	*NPM1, PCM1, SS18*
	I-O Biomarkers	
PD-L1 expression*	Not available	Not available
Tumor mutational burden (TMB)	Not available	3 mutations/Mb
Microsatellite instability (MSI)	Not available	Not available

*Assessed by immunohistochemistry (IHC)

**Archer® FusionPlex® assay; *TERT* promoter region was not covered by the analysis.

TMB = Tumor mutational burden; MSI = Microsatellite instability; CNA = Copy number amplifications (by NGS); I-O = Immuno-Oncology.

Table 1. Molecular findings in the Leydig cell tumors cohort

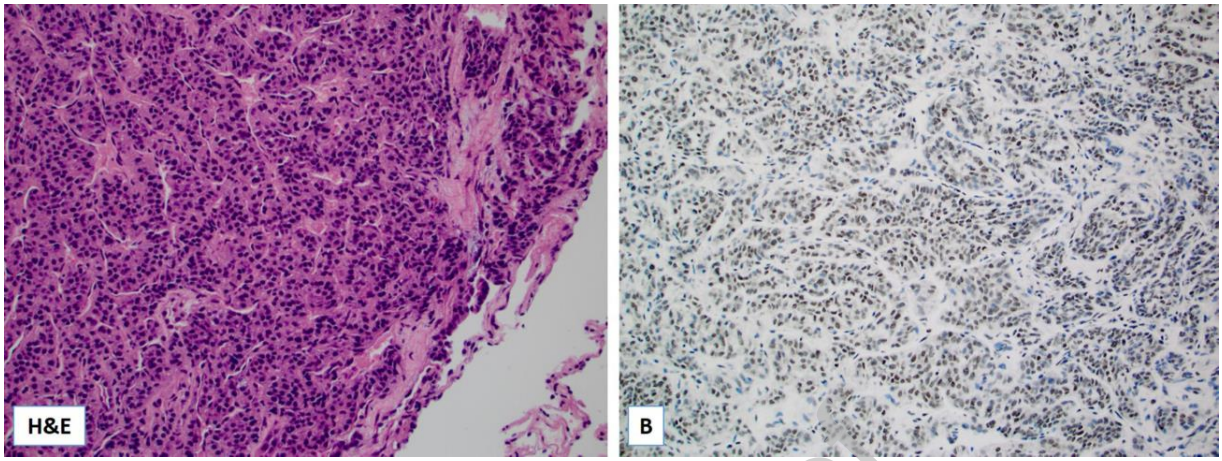

Figure 1. Hematoxylin and Eosin (H&E) slide of a metastatic Leydig cell tumor to the lung (A); the tumor cells were diffusely positive for Topo1 by immunohistochemistry (20x).

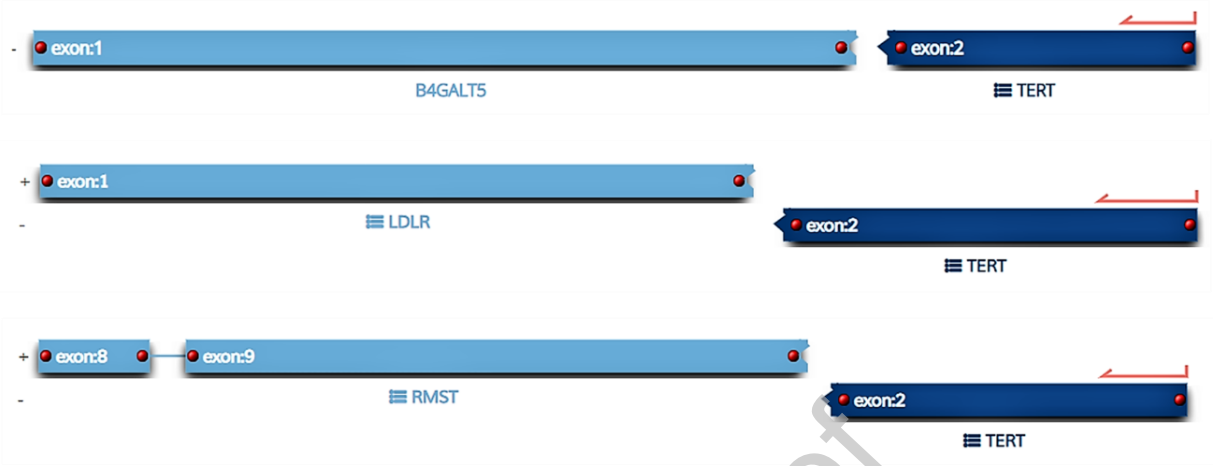

Figure 2. *TERT* gene fusions detected in three metastatic (malignant) Leydig cell tumors of the testis.