Testicular germ cell tumors type 2 have high RNA expression of LDHB, the gene for lactate dehydrogenase subunit B

Finn Edler von Eyben¹, Jorge Parraga-Alava²,³, Shi-Ming Tu⁴

This study analyzed RNA expression of genes for three serum tumor markers, alpha fetoprotein (AFP), human chorionic gonadotropin (hCG), and lactate dehydrogenase (LDH), in patients with testicular germ cell tumors (TGCT) type 2. The gene AFP encodes AFP, the gene for chorionic gonadotropin beta polypeptide 5 (CGB5) encodes a major part of the specific beta subunit of hCG, and the genes for LDH subunit A (LDHA), LDH subunit B (LDHB), and LDH subunit C (LDHC) encode three different subunits of LDH. LDHB encodes the LDHB subunit present as a tetramer in LDH isoenzyme 1 (LDH-1). We examined three datasets with 203 samples of normal testis tissue (NT) and TGCT type 2. Yolk sac tumor (YST) expressed RNA of CGB5 ten times higher than other histologic types of TGCT combined. EC expressed RNA of LDHB twice higher than SE, YST and TER combined (P = 0.000041). EC expressed RNA of LDHB higher than that YST expressed RNA of AFP and that CC expressed RNA of CGB5. In conclusion, TGCT type 2 expressed RNA of LDHB markedly higher than the RNA of 23 other candidate genes for TGCT type 2.

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INTRODUCTION

Current histopathologic classifications of testicular germ cell tumors (TGCT) type 2 of young adults have two main groups, seminoma (SE) and nonseminomatous germ cell tumors (NSGCT).¹ The TNM classification includes the serum tumor markers because they had prognostic significance in the International Germ Cell Consensus Group (IGCCCG) study.² The study stratified patients with metastatic TGCT into good-, intermediate-, and poor-risk groups. International guidelines follow the TNM classification.³ The serum tumor markers have discordant levels.⁴ For instance, 40% of patients with metastatic SE have elevated S-LDH and normal S-AFP and S-hCG.⁵

A recent cohort study including all Danish patients with TGCT type 2 from the modern era using a combination chemotherapy regimen with bleomycin, etoposide, and platinum (BEP) confirmed the prognostic significance of the IGCCCG classification.⁶ Another study showed that the serum tumor markers separated patients with intermediate risk into two groups: one had a prognosis like that of patients with good risk and another had a prognosis like that of the patients with poor risk.⁷

Two other studies showed that patients with poor risk who had limited decline of an initially raised S-AFP and S-hCG after the first cycles of BEP obtained an improvement of the overall survival if the patients were switched to a more aggressive form for chemotherapy than BEP.⁸,⁹

The gene AFP encodes AFP. hCG is characterized by a specific beta subunit, and six genes including chorionic gonadotropin beta polypeptide 5 (CGB5) encode the beta subunit of hCG. LDH consists of five LDH isoenzymes, and each LDH isoenzyme consists of four LDH subunits, either LDHA or LDHB or both.¹⁰ LDH isoenzyme 1 (LDH-1). We examined three datasets with 203 samples of normal testis tissue (NT) and TGCT type 2. Yolk sac tumor (YST) expressed RNA of CGB5 ten times higher than other histologic types of TGCT combined. EC expressed RNA of LDHB twice higher than SE, YST and TER combined (P = 0.000041). EC expressed RNA of LDHB higher than that YST expressed RNA of AFP and that CC expressed RNA of CGB5. In conclusion, TGCT type 2 expressed RNA of LDHB markedly higher than the RNA of 23 other candidate genes for TGCT type 2.

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isoenzyme 1 (LDH-1) is a tetramer of four LDHB subunits, and LDH isoenzyme 5 (LDH-5) is a tetramer of four LDHA subunits. Adult men have an additional LDH isoenzyme that is specific for testicular germ cells, LDH-X, which is a tetramer of four LDHC subunits. LDHA, LDHB, and LDHC encode the three LDH isoenzymes.

A recent meta-analysis reported how the histologic types of TGCT type 2 expressed RNA of 24 candidate genes for TGCT type 2 in three datasets. Complementarily, our present study aimed to elucidate whether the histologic types of TGCT type 2 differ in RNA expressions of AFP, CG85, LDHA, LDHB and LDHC, and to compare RNA expressions of genes for the serum tumor markers with RNA expressions of the 19 previously evaluated candidate genes.

MATERIALS AND METHODS

Materials

The present study evaluated three public available datasets of RNA expression of genes in TGCT type 2. The principal investigators for two of the datasets informed about the RNA expressions of 24 candidate genes in tissue samples of normal testis (NT) and TGCT type 2 from the individual patients. Thereby, the study included datasets of two microarray studies and an RNA dataset of a sequencing study.

The first microarray dataset was based on a German study, originally published in 2005. It included three samples of NT and 40 samples of SE. The study used Affymetrix HG-1195A version 2 (ThermoFisher Scientific, Waltham, MA, USA) to analyze RNA expression. The dataset is deposited at Gene Expression Omnibus (GEO) under the accession number GSE8607 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE8607).

The second dataset was based on a Norwegian microarray study originally published in 2005. It included three samples of NT, three samples of GCNIS, three samples of SE, five samples of EC, four samples of YST, four samples of TER, and one sample of CC. The study used Agilent Whole Human Genome 1A oligo microarray (Agilent Technologies, Santa Clara, CA, USA) to analyze RNA expression of genes.

Agilent’s Human 1A Oligo Microarray (version 2) includes over 17 000 60-mer oligonucleotide probes, sourced from the Incyte Foundation Database, and designed to span conserved exons across the transcripts of the targeted full-length genes. The second microarray dataset reported cDNA expression log, transformed relative to a reference standard of ten human cell lines.

The dataset of the second microarray is deposited at GEO under the accession number GSE 1818 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE1818).

The third dataset is part of the Cancer Genome Atlas (TCGA) project, originally published regarding the subgroup of patients with TGCT type 2 in 2018. The dataset included 72 samples of SE, 27 samples of EC, 13 samples of YST, and 16 samples of TER. The RNA expression was determined using RNA sequencing. The dataset is deposited at TCGA project available from https://portal.gdc.cancer.gov/legacy-archive/.

Selection of genes

Before the study was started, we determined to analyze 24 candidate genes for TGCT type 2: five genes for the serum tumor markers and 19 other genes. These candidate genes included six genes for pluripotency, Kruppel-like factor 4 (KLF4), LIN28 homolog A (LIN28A), MYC oncogene (MYC), homeobox transcription factor NANOG (NANOG), POU domain class 5, transcription factor 1 (POUSF1), and SRY-box 17 (SOX17), that were among the genes with the highest RNA expression in GCNIS.

The candidate genes also included protooncogenes that may be upregulated in TGCT type 2, such as cyclin D2 (CCND2), MDM2 protooncogene (MDM2), MYC, v-myc avian myelocytomatosis viral-related oncogene, neuroblastoma-derived (NMYC), pituitary tumor-transforming gene 1 (PTTG1), and cyclin E1 (CCNE1).

The candidate genes also included tumor suppressor genes that may be downregulated in TGCT type 2, such as cyclin-dependent kinase inhibitor 1A (CDKN1A), cyclin-dependent kinase inhibitor 2C (CDKN2C), phosphatase and tensin homolog (PTEN), and retinoblastoma corepressor 1 (RB1). The candidate genes also included preferentially expressed antigen in melanoma (PRAME), a biomarker that is highly expressed in SE, and DNA methyltransferase 3B (DNMT3B), a biomarker that is highly expressed in EC. The candidate genes also included forkhead box D (FOXOD).

Definitions and methods

A gene was defined to express RNA significantly in a histologic type of TGCT type 2 if only the single histologic type of TGCT type 2 expressed RNA of the candidate genes insignificantly. A gene was defined as a genetic signature of a histologic type of TGCT type 2 if only the single histologic type characteristically and highly expressed RNA of the gene.

Our study summarized RNA expressions of the candidate genes in samples of NT and the histologic types of TGCT type 2. We evaluated whether NT and the histologic types of TGCT type 2 expressed RNA of the candidate genes differently and whether undifferentiated histologic types of TGCT type 2, SE and EC, and differentiated histologic types of NSGCT expressed RNA of the candidate genes differently. Further, we examined whether TGCT type 2 expressed RNA of genes for the serum tumor markers in the tumor lesions different from RNA expression of the 19 comparator genes.

We did not substitute missing data regarding RNA expression of a gene in the datasets. The null hypothesis of the study was that all TGCT type 2 only expressed RNA of the candidate genes insignificantly. Hence, we gave priority to comparisons between single histologic types or groups of histologic types that differed significantly in RNA expression of the genes for the serum tumor markers.

Statistical analyses

We had focus on the median RNA expression of the genes as we summarized the RNA expression of the genes and analyzed whether the histologic types expressed RNA of the genes differently, using Kruskal–Wallis tests. We calculated a meta-analytic P value using the Fisher’s test as we analyzed the three datasets regarding RNA expressions of genes for the serum tumor markers.

We carried out the statistically two-tailed analyses and considered P < 0.05 as statistically significant. The statistical analyses were carried out using Stata version 14.2 (Stata Corp, College Station, TX, USA).

RESULTS

Genes for the serum tumor markers

Our study included three datasets with 203 samples of NT and TGCT type 2. Table 1 shows clinical characteristics of the patients with TGCT type 2 in the TCGA study. Overall, our study evaluated six samples of NT, three samples of GCNIS, 115 samples of SE, and 79 samples of NSGCT. In the three datasets, the RNA expression of the
five genes for the serum tumor markers differed concordantly between the histologic types.

YST was associated with AFP (Figure 1). In the first microarray, NT and SE did not differ significantly regarding RNA expression of AFP (Figure 1a). In the second microarray, YST expressed RNA of AFP five hundred times higher than NT (94 vs 0.17, \(P = 0.06\), Kruskal–Wallis test, Figure 1b). In the TCGA dataset, YST expressed RNA of AFP fourteen thousand times higher than SE, EC, and TER combined (median: 12 883.1 vs 0.9, \(P = 0.0001\), Kruskal–Wallis test, Figure 1c).

Especially YST had a high RNA expression of AFP. Some TER had a relatively high RNA expression of AFP likely to be due to undiagnosed YST elements within the TER. Hence, we consider AFP to be a genetic signature of YST.

CC was associated with GBS5 (Figure 2). In the first microarray, SE had slightly higher RNA expression of GBS5 than NT (Figure 2a). In the second microarray, CC expressed RNA of GBS5 nine times higher than NT, but the difference was not statistically significant (9.0 vs 1.0, \(P = 0.15\), Kruskal–Wallis test, Figure 2b). CC expressed RNA of GBS5 ten times higher than the other histologies of TGCT combined, but the difference was not statistically significant (9.0 vs 0.9, \(P = 0.10\), Kruskal–Wallis test).

In the first microarray, SE overall had a slightly raised RNA expression of GBS5 that may be due to the presence of syncytiotrophoblastic cells in some of the SE. In the second microarray, only CC had a high RNA expression of GBS5. Hence, we consider GBS5 to be a genetic signature of CC.

EC was associated with LDHB (Figure 3). In the first microarray, SE had significantly higher RNA expression of LDHB than NT (\(P = 0.002\), Kruskal–Wallis test, Figure 3a). In the second microarray, EC expressed RNA of LDHB six times higher than NT (2.4 vs 0.36, \(P = 0.025\), Kruskal–Wallis test, Figure 3b). In the TCGA dataset, EC expressed RNA of LDHB twice higher than SE, YST, and TER combined (median: 45 154 vs 18 708, \(P = 0.0001\), Kruskal–Wallis test, Figure 3c).

In the first microarray, NT and SE did not differ significantly in RNA expression of LDHA (\(P = 0.28\), Kruskal–Wallis test). In the second microarray, EC expressed RNA of LDHA twice higher than NT, but the difference was not statistically significant (1.7 vs 0.82, \(P = 0.25\), Kruskal–Wallis test). In the TCGA dataset, SE and EC combined expressed RNA of LDHA twice higher than YST and TER combined, but the difference was not statistically significant (median: 19 977 vs 6897, \(P = 0.06\), Kruskal–Wallis test).

In the first microarray, NT expressed RNA of LDHC significantly higher than SE (\(P = 0.004\), Kruskal–Wallis test). In the second microarray, NT expressed RNA of LDHC 53 times higher than SE (median: 53 vs 1.0, \(P = 0.0495\), Kruskal–Wallis test). Both NT and GCNIS had markedly higher RNA expression of LDHC than SE, EC, TER, and YST combined.

Some GCNIS had a relatively high RNA expression of LDHC that is likely to be due to undiagnosed NT elements in the GCNIS. Hence, we consider LDHC to be a genetic signature of NT.

In the TCGA dataset, AFP and LDHB were among the genes that had the highest RNA expression in YST and EC, respectively. EC expressed RNA of LDHB higher than RNA of LDHA. Importantly, EC had a higher median and maximum RNA expression of LDHB than that YST had of AFP.

**Comparative genes**

In the previous meta-analysis of the three datasets, EC relatively highly expressed RNA of LIN28A, CCND2, and NANOG. \(^4\) Nevertheless in the TCGA dataset in our present study, EC expressed RNA of LDHB higher than RNA of ten significant comparative genes (Figure 4).

**Table 1:** Clinical characteristics of patients in the cancer genomic atlas project study

| Characteristics                        | Values          |
|----------------------------------------|-----------------|
| Age at diagnosis (year), median (range) | 31 (14–66)      |
| Stage of TGCT (n)                      |                 |
| I                                      | 14              |
| IA                                     | 27              |
| IB                                     | 10              |
| IS                                     | 42              |
| II                                     | 4               |
| IIA                                    | 11              |
| IIB                                    | 1               |
| IIC                                    | 1               |
| III                                    | 1               |
| IIAA                                   | 1               |
| IIBA                                   | 8               |
| IIBB                                   | 8               |
| IIC                                    | 5               |

Preoperative S-AFP (U ml\(^{-1}\), median (range)) 5.4 (0.9–37 822)

**Table 2:** Meta-analysis of the three datasets of histologic types of testicular germ cell tumor type 2 regarding \(P\) values for RNA expression of genes for the serum tumor markers

| Gene       | Comparison of histologic types | Number of included datasets | \(P\) values in the meta-analyses of the datasets |
|------------|-------------------------------|-----------------------------|-----------------------------------------------|
| AFP        | Y vs SET                       | 2                           | 0.00015                                       |
| LDHB       | N vs S                         | 2                           | 0.00015                                       |
| LDHB       | E vs SYT                       | 2                           | 0.000041                                      |
| LDHA       | N vs S                         | 2                           | 0.23                                          |
| LDHA       | E vs YT                        | 2                           | 0.00021                                       |
| LDHC       | N vs S                         | 2                           | 0.0019                                        |

**Meta-analysis of RNA expression in the histologic types of genes for the serum tumor markers**

Meta-analyses of the three datasets that combined the \(P\) values for RNA expression of genes for the serum tumor markers in histologic types are shown in Table 2. YST expressed RNA for AFP significantly higher than SE, EC, and TER combined. SE expressed RNA of LDHA significantly higher than NT, whereas SE did not express RNA of LDHA significantly higher than NT.

**DISCUSSION**

The study is an in silico investigation that increases the knowledge regarding the background for the three serum tumor markers of TGCT type 2. The study demonstrated that EC expressed RNA of LDHB markedly higher than RNA of 23 other candidate genes for TGCT type 2. The findings explain why many patients with SE and EC have a raised S-LDH, whereas mainly patients with elements of YST and CC in NSCST have raised S-AFP and raised S-hCG. Our LDHB findings link a high S-LDH and a high S-LDH-1 in patients with TGCT type 2 to a high copy number of the short arm of chromosome 12 (12p), in the malignant germ cells, often in the form of a characteristic isochromosome, i(12p). \(^{10,31}\)
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The gene loci for AFP (4q13.3) and CGB5 (19q13.32) are not located on 12p. YST does not have an increased copy number of chromosome 4q. However, the two histologic types of differentiated NSGCT highly expressed RNA of specific genes for AFP and hCG. YST highly expressed RNA of specific genes for AFP and hCG. Both S-LDH and total tumor volume were significant prognostic factors.35

Copy number of 12p and RNA expression of LDHB in TGCT type 2 lesions were associated with the levels of S-LDH/S-LDH-1 in previous studies.14,35 In patients with TGCT type 2, the product of the overall tumor mass and the copy number of 12p in malignant germ cells in cytogenetic analyses of TGCT type 2 correlated significantly with S-LDH-1.34 In nude mice with transplants of human TGCT type 2, the product of the TGCT mass and the RNA expression of LDHB in tumor lesions correlated markedly with S-LDH/S-LDH-1.35 Patients with TGCT type 2 and a raised S-LDH have a high proportion of LDH-1.33-37 The TCGA dataset in our present study showed that EC expressed RNA of LDHB higher than the RNA of CCND2 and NANOG. The previous meta-analysis of the 24 candidate genes for TGCT type 2 in the three datasets considered CCND2 and NANOG to be driver genes for TGCT type 2.36 Hence, due to the
relatively high RNA expression, our study indicates that also LDHB is a driver gene for TGCT type 2.

The high RNA expression of LDHB is the genetic background for patients with TGCT type 2 and a raised S-LDH have a characteristic high proportion of S-LDH-1.\(^{36,37}\) Both a systematic review and a critical narrative review document that S-LDH-1 is a relevant serum tumor marker for patients with both SE and EC.\(^{5,38}\) Adding S-LDH-1 to the TNM classification increased the prognostic prediction for patients with TGCT type 2.\(^{39}\) LDHB has major biologic effects. Knock-down of LDHB in lung adenocarcinoma cells reduced growth of the cells.\(^{40}\) Knock-down of LDHB also reduced cell proliferation of maxillary sinus squamous cancer cells.\(^{41}\) LDHB also has important roles for other malignancies.\(^{32,42}\)

The dramatic effects of expression of LDHB reflect that LDH has a central role for anaerobic glycolysis. Anaerobic glycolysis metabolizes intracellular glucose to pyruvate and LDH metabolizes the resulting pyruvate to lactate.\(^{44}\) Thus, LDH helps malignant cells in the initial steps of glycolysis to produce adenosine 5-triphosphate (ATP) without need of oxygen. Hence, a high LDH activity in malignant cells gives the malignant cells a survival advantage in a hypoxic environment. The Warburg effect\(^{45}\) explains why more patients with TGCT type 2 and a raised S-LDH die of TGCT than patients with a normal S-LDH.\(^{46}\)

Our study shows that TGCT type 2 expresses RNA of LDHB higher than RNA of LDHA and LDHC. RNA expression of LDHA, LDHB, and LDHC is regulated by epigenetic mechanisms.\(^{46}\) In a retinoblastoma cell line, a complete methylation of the promoter for LDHA blocked expression of LDHA\(^{47}\) and demethylation with 5-aza-2-deoxycytidine restored RNA expression of LDHA, and thereby production of LDHA in the cell line.

Epigenetic mechanisms downregulate LDHC in most normal tissues. Methylation of the promoter of LDHC inhibits RNA expression of LDHC. Transcription factor Sp1 (Sp1) and cAMP responsive element inducing protein 1 (CREB1) regulated gene expression of LDHC.\(^{48}\) Neurofibromin 1 (NF-1) blocked RNA expression of LDHC.\(^{49}\) Correspondingly, adult men have LDH-X in normal testicular germ cells, but S-LDH-X is not detectable in blood.\(^{50}\) Our study shows that TGCT type 2 downregulates LDHC. The downregulation of LDHC explains why patients with TGCT type 2 and raised S-LDH have no detectable S-LDH-X.\(^{37,31}\)

The 12p has a crucial role for the development of TGCT type 2.\(^{30}\) SE and NSGCT have an increased copy number of 12p, often as an isochromosome i(12p).\(^{30,34}\) The analysis of the RNA expression of the 24 genes in the previous meta-analysis and in our present study of the three datasets elucidates that the crucial role of 12p in TGCT type 2 is due to specific genes on 12p.\(^{36,37}\) CCND2, NANOG, and LDHB have gene loci on 12p: 12p13.32, 12p13.31, and 12p12.1, respectively; and LDHB have gene loci close to a candidate ampiclon of 12p.\(^{35}\) Our study shows that TGCT type 2 had higher RNA expression of LDHB than that of CCND2 and NANOG. Likewise, previous publications of genes on 12p in TGCT type 2 showed high RNA expression of LDHB.\(^{35,34}\) Hence, the RNA expression of LDHB is not solely regulated by the copy number of 12p in the tumors. One of the previous publications also showed that YST highly expressed RNA of LDHB.

In 2019, a systematic review claimed that there is no evidence for the use of S-LDH in combination with S-AFP and S-hCG in any type of TGCT.\(^{39}\) However, multivariate analyses of the serum tumor markers in a study of patients with intermediate risk showed that both S-AFP and S-LDH were significant predictors of progression free survival and of overall survival.\(^{42} \) Zondag and Klein\(^{45}\) previously found that SE had a unique S-LDH isoenzyme pattern with a predominant S-LDH-1.

Correspondingly, future analyses of the TCGA project may investigate whether SE and EC have higher RNA expression of LDHB than all other cancer types. In addition, our findings motivate future studies of cell lines of TGCT type 2 where LDHB is blocked in the malignant germ cells. Our findings also argue for further clinical studies of S-LDH and S-LDH-1 as serum tumor markers of patients with TGCT type 2. Our study has limitations. It is not a systematic review. Recent guidelines do not include S-LDH-1 as a serum tumor marker of TGCT type 2.\(^{36}\)

No study has analyzed whether posttranslational modifications of LDHB subunits enhance the catalytic activity of LDH observed in other studies.\(^{56}\)

**CONCLUSIONS**

TGCT type 2 has a markedly high RNA expression of LDHB, the genetic background for LDH-1. Differences in RNA expressions of AFP, CGB5, and LDHB in the histologic types contribute to discordant levels of the serum tumor markers in patients with TGCT type 2. Our LDHB findings support the recommendation for patients with TGCT type 2 with a raised S-LDH that measurements of S-LDH-1 help to determine whether the raised LDH activity is related to the tumors.\(^{36,37}\)

**AUTHOR CONTRIBUTIONS**

FeV\(^{\circ}\)E made the concept for the study, made statistical analyses, and wrote the first draft of the manuscript. JPA and SMT separately performed realanalyses of the first microarray dataset and contributed actively to the revisions of the manuscript. JPA examined gene expression in data files. All authors read and approved the final manuscript.

**COMPETING INTERESTS**

All authors declare no competing interests.

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