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## **Title: FOXA2 is a reliable marker for the diagnosis of yolk sac tumor postpubertal-type**

Costantino Ricci (ORCID: 0000-0001-7254-4195)<sup>1,2</sup>, Francesca Ambrosi (ORCID: 0000-0001-9046-1115)<sup>1,2</sup>, Tania Franceschini (ORCID: 0000-0003-2897-7756)<sup>1</sup>, Francesca Giunchi (ORCID: 0000-0001-5298-939X)<sup>3</sup>, Giorgia Di Filippo<sup>1</sup>, Eugenia Franchini<sup>1</sup>, Francesco Massari (ORCID: 0000-0001-6476-6871)<sup>2,4</sup>, Veronica Mollica (ORCID: 0000-0002-5169-3631)<sup>2,4</sup>, Valentina Tateo (ORCID: 0000-0002-1199-2338)<sup>2,4</sup>, Federico Mineo Bianchi (ORCID: 0000-0003-3331-0374)<sup>5</sup>, Maurizio Colecchia (ORCID: 0000-0003-1914-0743)<sup>6</sup>, Andres Martin Acosta (ORCID: 0000-0003-0164-5911)<sup>7</sup>, Michelangelo Fiorentino (ORCID: 0000-0002-1749-150X)<sup>1,2</sup>

<sup>1</sup>Pathology Unit, Maggiore Hospital-AUSL Bologna, Bologna, Italy;

<sup>2</sup>Department of Medical and Surgical Sciences (DIMEC), University of Bologna, Bologna, Italy;

<sup>3</sup>Pathology Unit, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Bologna, Italy;

<sup>4</sup>Medical Oncology, IRCCS Azienda Ospedaliero-Universitaria di Bologna, Bologna, Italy;

<sup>5</sup>Urology Department, Maggiore Hospital-AUSL Bologna, Bologna, Italy;

<sup>6</sup>Department of Pathology, IRCCS San Raffaele Scientific Institute, Milano, Italy;

<sup>7</sup>Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, USA;

*Corresponding Author:* Costantino Ricci; Department of Medical and Surgical Sciences (DIMEC), University of Bologna, Via Massarenti 9, Bologna-40138, Italy; e-mail: costantino.ricci4@unibo.it;

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## **Abstract**

*Aims:* Yolk sac tumor postpubertal-type (YSTpt) shows a wide range of histologic patterns and is challenging to diagnose. Recently, forkhead box transcription factor A2 (FOXA2) emerged as a driver of YSTpt formation and a promising marker for diagnosing YSTpt. However, FOXA2 has not been tested in the different patterns of YSTpt. The study aimed to assess the staining pattern of FOXA2 in the different patterns of YSTpt and other germ cell tumors of the testis (GCTT), comparing it with glypican-3 (GPC3) and  $\alpha$ -fetoprotein (AFP).

*Methods and results:* FOXA2, GPC3, and AFP immunohistochemistry was performed on 24 YSTpt (24 microcystic/reticular, 10 myxoid, 2 macrocystic, 5 glandular/alveolar, 2 endodermal sinus/perivascular, 4 solid, 2 polyembryoma/embryoid body, and 2 polyvesicular vitelline) and 81 other GCTT. The percentage of positive cells (0, 1+, 2+, 3+) and the intensity (0, 1, 2, 3) were evaluated regardless of and within each YSTpt pattern. FOXA2 was positive in all YSTpt (24/24) and all but one (23/24) exhibited 2+/3+ stain, with higher intensity [median value (mv): 2.6] than AFP (1.8) and GPC3 (2.5). Both FOXA2 and GPC3 were positive in all microcystic/reticular (24/24), myxoid (10/10), macrocystic (2/2), endodermal sinus/perivascular (4/4), and polyembryoma/embryoid body (2/2) patterns. Nevertheless, only FOXA2 was positive in all glandular/alveolar (5/5), solid (4/4), and polyvesicular vitelline (2/2) patterns. The intensity of FOXA2 was higher than AFP and GPC3 in almost all YST patterns. In the other GCTT, FOXA2 was positive only in teratoma postpubertal-type (Tpt) [13/20 (65%)], with staining almost exclusively confined to the mature gastrointestinal/respiratory tract epithelium.

*Conclusions:* FOXA2 is a highly sensitive and specific biomarker that supports the diagnosis of YSTpt. FOXA2 is superior to GPC3 and AFP, especially in rare and difficult-to-diagnose histologic patterns of YSTpt, but mature glands of Tpt could represent a potential diagnostic pitfall.

**Keywords:** FOXA2, Glypican 3,  $\alpha$ -fetoprotein, yolk sac tumor, yolk sac tumor postpubertal-type, germ cell tumors of the testis;

**Abbreviations:** forkhead box transcription factor A2 (FOXA2), forkhead box gene (*FOX*), seminoma/seminomas (S), nonseminomatous germ cell tumors of the testis (NSGCTT), yolk sac tumor/tumors (YST), SRY-box 17 (SOX17), glypican-3 (GPC3),  $\alpha$ -fetoprotein (AFP), primary mediastinal germ cell tumors (PMGCT), germ cell tumors of the testis (GCTT), hematoxylin and eosin (H&E), yolk sac tumor/tumors postpubertal-type (YSTpt), embryonal carcinoma/carcinomas (EC), teratoma/teratomas postpubertal-type (Tpt), isolated syncytiotrophoblast cells (iSTCs), choriocarcinoma/choriocarcinomas (CHC), somatic-type malignancy/malignancies with the features of embryonic-type neuroectodermal tumor (STM ETNT), somatic-type malignancy/malignancies with the features of rhabdomyosarcoma (STM RMS), somatic-type malignancy/malignancies with the features of sarcoma not otherwise specified (STM Snos), germ cell neoplasia in situ (GCNIS), sal-like protein 4 (SALL4), octamer-binding transcription factor 4 (OCT4), cluster of differentiation 117/tyrosine-protein kinase kit (CD117/c-kit), cluster of differentiation 30 (CD30), human choriogonadotropin (h-GC), cytokeratins cocktail AE1/AE3 (CK AE1/AE3), SRY-box 2 (SOX2), transcription factor encoded by *GATA3* (GATA3), homeobox protein CDX-2 (CDX2), hepatocyte nuclear factor 1 homeobox B (HNF1 $\beta$ ), polymerase chain reaction (PCR).

## Introduction

Forkhead box transcription factor A2 (FOXA2) belongs to the forkhead box gene (*FOX*) superfamily.<sup>1-5</sup> It is a pivot molecule in embryonic development that regulates the formation of the notochord, nervous system, and several endodermal structures.<sup>1-5</sup> In recent years, FOXA2 proved to be crucially involved in several metabolic processes (bile acids and lipids homeostasis, clearance of fatty acids, response to insulin, and many others) and its expression has been demonstrated in many tumor types (neuroendocrine lung and prostate tumors, colorectal carcinoma, breast carcinoma, melanoma, and many others).<sup>1-12</sup> *Nettersheim D et al.* found that FOXA2 plays a key role in the progression from seminoma (S) to nonseminomatous germ cell tumors of the testis (NSGCTT), the so-called “reprogramming” of S cells.<sup>13-16</sup> Specifically, FOXA2 regulates the expression of genes associated with yolk sac tumor (YST) differentiation [SRY-box 17 (SOX17), glypican-3 (GPC3),  $\alpha$ -fetoprotein (AFP), and others] and is a putative effector of the YST phenotype.<sup>13-16</sup> The same authors found a strong nuclear expression of FOXA2 in all tested cases (100%) of adult and pediatric YST, being more sensitive of GPC3 and AFP.<sup>16</sup> *Fichtner A et al.* tested a case series of primary mediastinal germ cell tumors (PMGCT) and found that all YST in their study (4/4) were diffusely positive for FOXA2.<sup>17</sup> In these studies, evaluation of the differential expression of FOXA2 in the different histologic patterns of YST and quantitative assessments of FOXA2 staining were not performed.<sup>16,17</sup> Differentiating the subtypes of germ cell tumors of the testis (GCTT) may be challenging on hematoxylin and eosin (H&E) slides alone.<sup>18,19</sup> In particular, testicular YST postpubertal-type (YSTpt) comprises multiple histologic patterns that can be present alone or in combination.<sup>18,19</sup> Understanding the sensitivity and specificity of FOXA2 in the different histologic patterns of YSTpt and other GCTT is the first step to properly compare the performance of this stain to that of established YST markers (GPC3 and AFP).

In the present study, we performed a quantitative and qualitative evaluation (percentage of positive cells and intensity) of FOXA2 expression in a cohort of testicular YSTpt and other GCTT (percentage of positive cells and intensity) comparing this marker to GPC3 and AFP.

## **Materials and methods**

### **Case series**

We queried our institutional database (Pathology Unit, Maggiore Hospital-AUSL Bologna, Bologna) for YSTpt diagnosed between January 1<sup>st</sup> 2012 and December 1<sup>st</sup> 2022. Fifteen additional GCTT with no YSTpt component (see below for more details) were retrieved and evaluated. Clinical parameters (age and tumor size) were electronic medical records of the Urology Department, Maggiore Hospital-AUSL Bologna. All cases had been diagnosed according to the 5<sup>th</sup> edition of the WHO classification of urinary and male genital tumors.<sup>19</sup> All the cases in this cohort have been previously published by our group.<sup>20-22</sup>

### **Immunohistochemistry**

All cases were reviewed to confirm the original diagnosis, assess the histologic patterns, and select a representative block. Three consecutive 3- $\mu$ m sections were cut from each paraffin-embedded tissue block and stained with the following antibodies: AFP, GPC3, and FOXA2 (BenchMark ULTRA automated immunostainer; Ventana Medical Systems-Roche Diagnostics, Switzerland). Detailed information of the antibodies used for immunohistochemistry is provided in *Supplementary Material 1*. Slides were incubated in H<sub>2</sub>O<sub>2</sub> and C<sub>2</sub>H<sub>6</sub>O for 30 min to block endogenous peroxidase activity and subsequently incubated with primary antibodies (1 hour at room temperature) and rabbit anti-mouse secondary antibody. The OptiView DAB IHC Detection Kit (Ventana Medical Systems-Roche Diagnostics, Switzerland) was used as the chromogen, and an intermediate step of amplification with OptiView Amplification Kit (OptiView Amplifier, OptiView Amplification H<sub>2</sub>O<sub>2</sub>, and OptiView Amplification Multimer; Ventana Medical Systems-



Roche Diagnostics, Switzerland) was used for FOXA2. Additionally, developed and standardized in-house and commercial positive control tissues were adopted for AFP (placenta), GPC3 (hepatocarcinoma), and FOXA2 (colo-rectal mucosa). Slides stained for AFP, GPC3, and FOXA2 were read simultaneously by two dedicated uropathologists (M.F. and C.R.) on a multi-head microscope and expression was assessed as previously described.<sup>16,17,23</sup> FOXA2 (nuclear), GPC3, and AFP (cytoplasmic and membranous stain) were semi-quantitatively evaluated as follows: negative (0, <5% of stained cells), focally positive (1+, 5-10% of stained cells), moderately positive (2+, 11-50% of stained cells), or diffusely positive (3+, >50% of stained cells).<sup>23</sup> The intensity of immunoreactivity was scored from 0 to 3 (no stain: 0, weak: 1, moderate: 2, strong: 3) for AFP, GPC3, and FOXA2, and the mean value (mv) was calculated.<sup>23</sup> Staining characteristics were assessed as GCTT-wide variables (i.e., percent of positive cells and average intensity in singly-evaluated GCTT), histologic pattern-specific variables for YSTpt (i.e., percent of positive cells and average intensity within each histologic pattern of YSTpt), and within germ cell neoplasia in situ (GCNIS).<sup>23</sup> Comparative evaluation of H&E and pertinent stains [sal-like protein 4 (SALL4), octamer-binding transcription factor 4 (OCT4), cluster of differentiation 117/tyrosine-protein kinase kit (CD117/c-kit), cluster of differentiation 30 (CD30), human choriogonadotropin (h-GC), cytokeratins cocktail AE1/AE3 (CK AE1/AE3), and SRY-box 2 (SOX2)] were assessed in parallel to correctly score AFP, GPC3 and FOXA2 expression in YSTpt, other GCTT, and GCNIS.

### **Ethics Committee**

All clinical-pathological investigations were conducted according to the principles of the Declaration of Helsinki and all information regarding the human material used in this study has been managed using anonymous numerical codes. The study has been approved by the local ethics committee/CE-AVEC Bologna-Emilia Romagna (463-2022-AUSLBO-22092-ANAPAT TESTIS 03).

## Results

### Case series

We retrospectively collected 24 YSTpt [1/24 (4.2%) pure and 23/24 (95.8%) components of mixed GCTT]; in the subgroup of YSTpt mixed with other GCTT, the percentage of YSTpt component ranged from 5% to 70% (mean value: 26%). The histologic patterns of YSTpt identified in this series included: microcystic/reticular, n = 24/24 (100%); myxoid, n = 10/24 (41.7%); macrocystic, n = 2/24 (8.3%); glandular/alveolar, n = 5/24 (20.8%); endodermal sinus/perivascular, n = 2/24 (8.3%); solid, n = 4/24 (16.7%); polyembryoma/embryoid body, n = 2/24 (8.3%); polyvesicular vitelline, n=2/24 (8.32%). The mean age at diagnosis was 32.3 years (range: 14-55 years) and the mean tumor size was 5 cm (range: 2-8.9 cm). Fifteen additional GCTT with no YSTpt component were collected, and the total of other GCTT components analyzed was 81 [56/81 (69.1%) obtained from the 23 mixed YSTpt and 25/81 (30.9%) from the 15 additional GCTT with no YSTpt component], as follows: 14/81 (17.3%) S, 24/81 (29.6%) embryonal carcinomas (EC), 20/81 (24.7%) teratomas postpubertal-type (Tpt), 10/81 (12.3%) isolated syncytiotrophoblast cells (iSTCs), 5/81 (6.2%) choriocarcinomas (CHC), 2/81 (2.5%) somatic-type malignancies with the features of embryonic-type neuroectodermal tumor (STM ETNT), 1/81 (1.2%) somatic-type malignancy with the features of rhabdomyosarcoma (STM RMS), and 1/81 (1.2%) somatic-type malignancy with the features of sarcoma not otherwise specified (STM Snos). Foci of GCNIS were detected in 26/39 (66.7%) cases in the sections chosen for immunohistochemistry. Clinico-pathologic data and immunohistochemistry results are summarized in *Supplementary Materials 2* and *3*.

### AFP

Most YSTpt cases expressed AFP [23/24 (95.8%)] but the staining pattern was often focally and/or moderately positive [0: 1/24 (4.2%), 1+: 16/24 (66.7%), 2+: 5/24 (20.8%)], with an intensity mv of 1.8 (*Supplementary Material 2*). AFP was positive in almost all microcystic/reticular and myxoid [21/24 (87.5%) and 9/10 (90%), respectively] patterns, but only 2/24 (8.3%) microcystic/reticular

patterns showed diffuse staining (3+). AFP stained all macrocystic [2/2 (100%)], endodermal sinus/perivascular [2/2 (100%)], and polyembryoma/embryoid body [2/2 (100%)] patterns; in contrast, AFP was positive in 3/5 (40%) glandular/alveolar, 1/4 (25%) solid, and 0/2 (0%) polyvesicular patterns. AFP was positive in 26/81 (32.1%) other GCTT components but the staining pattern was often focally and/or moderately positive [1+: 17/81 (21%), 2+: 8/81 (9.9%)], with an intensity mv of 0.5 (*Supplementary Material 3*). Among the other GCTT components, Tpt showed the highest values of AFP expression [1+: 7/20 (35%), 2+: 5/20 (25%), with an intensity mv of 1], mainly detected in the mature gastrointestinal/respiratory tract epithelium (glands and cysts). No GCNIS cases [0/26, (0%)] turned out positive for AFP. AFP showed diffuse background reactivity, especially in the hemorrhagic areas, necrosis, and cystic-glandular fluid secretions. AFP staining in the different histologic patterns of YSTpt and other GCTT components is summarized in *Table 1*.

### GPC3

All cases of YSTpt were positive for GPC3 [24/24 (100%)], and all but two cases exhibited moderate and/or diffuse staining [2+ and 3+: 22/24 (91.7%)] with an intensity mv of that was higher than that of AFP (2.5 vs. 1.8) (*Supplementary Material 2*). GPC3 was positive in all microcystic/reticular [24/24 (100%)] and all but one myxoid [9/10 (90%)] patterns, with diffuse positivity (3+) in most of the cases [17/24 (70.8%) microcystic/reticular and 6/10 (60%) myxoid]. GPC3 stained all macrocystic [2/2 (100%)], endodermal sinus/perivascular [2/2 (100%)], and polyembryoma/embryoid body [2/2 (100%)] patterns; in contrast, GPC3 was positive in 4/5 (80%) glandular/alveolar, 3/4 (75%) solid, and 1/2 (50%) polyvesicular patterns. The mv of intensity was higher for GPC3 than AFP in all histologic patterns, except for endodermal sinus/perivascular (mv: 1). GPC3 was positive in 50/81 (61.7%) other GCTT components with the staining pattern ranging from focally to diffusely positive [1+: 21/81 (25.9%), 2+: 26/81 (32.1%), 3+: 3/81 (3.7%)], and an intensity mv of 1 (*Supplementary Material 3*). Among the other GCTT

components, CHC showed the highest values of GPC3 expression [1+: 2/5 (40%), 2+: 3/5 (60%), with an intensity mv of 2], mainly detected in syncytiotrophoblast rather than cytotrophoblast and intermediate trophoblast cells. Other GCTT with significant GPC3 were EC [1+: 8/24 (33.3%), 2+: 8/24 (33.3%), and 3+: 1/24 (4.2%), with an intensity mv of 1.1 and the expression observed in both epithelium and stroma (neoplastic and non-neoplastic type)], Tpt [1+: 7/20 (35%), 2+: 7/20 (35%), and 3+: 1/20 (5%), with an intensity mv of 1 and the expression mainly detected in the mature gastrointestinal/respiratory tract epithelium (glands and cysts) and the fibroblastic/myofibroblastic stroma], and iSTCs [1+: 3/14 (21.4%), 2+: 3/14 (21.4%), with an intensity mv of 0.8]. Moreover, relevant GPC3 expression was also observed in STM, with the only STM RMS case showing diffuse and moderate GPC3 expression [3+: 1/1 (100%), and intensity mv of 2]. No GCNIS cases [0/26, (0%)] turned out positive for GPC3. GPC3 showed little background reactivity, artefactual/nonspecific staining largely restricted to necrotic areas and cystic-glandular fluid secretions. GPC3 staining in the different histologic patterns of YSTpt and other GCTT components is summarized in *Table 2*.

## **FOXA2**

All cases of YSTpt were positive for FOXA2 [24/24 (100%)], and all but one case exhibited moderate and/or diffuse staining [2+ and 3+: 23/24 (95.8%)] with an intensity mv that was higher than that of AFP and GPC3 (2.6 vs. 1.8 and 2.5) (*Supplementary Material 2*). FOXA2 was positive in all microcystic/reticular [24/24 (100%)] and myxoid [10/10 (90%)] components, with the vast majority of these cases exhibiting a diffuse positivity (3+) [19/24 (79.2%) and 7/10 (70%), respectively]. FOXA2 stained all macrocystic [2/2 (100%)], endodermal sinus/perivascular [2/2 (100%)] polyembryoma/embryoid body [2/2 (100%)], glandular/alveolar [5/5 (100%)], polyvesicular [2/2 (100%)], and solid [4/4 (100%)] components. The intensity mv of FOXA2 was higher than that of AFP in all histologic patterns; the intensity mv of FOXA2 was higher than that

of GPC3 in histologic patterns except for macrocystic (mv: 3) and polyembryoma/embryoid body (mv: 3). Among the other GCTT components, FOXA2 was positive only in Tpt [13/81 (16.1%) other GCTT components, 13/20 (65%) Tpt], with a staining pattern focally and/or moderately positive [1+: 4/20 (20%), 2+: 9/20 (35%)], an intensity mv of 1.2 (*Supplementary Material 3*), and the expression mainly observed in the mature gastrointestinal/respiratory tract epithelium (glands and cysts). No GCNIS cases [0/26, (0%)] turned out positive for FOXA2. FOXA2 did not show background reactivity, with an easily interpretable nuclear signal. FOXA2 staining in the different histologic patterns of YSTpt and other GCTT is summarized in *Table 3*.

Illustrative examples of AFP, GPC3, and FOXA2 stains in the different histologic patterns of YSTpt are shown in *Figures 1* and *2*, and *3*.

## **Discussion**

In the 5<sup>th</sup> edition of the WHO classification of urinary and male genital tumors, YSTpt is defined as a GCTT that recapitulates structures of the embryonic yolk sac, allantois, and extraembryonic mesenchyme.<sup>18</sup> Accurate identification of YSTpt, even when present as relatively small foci in mixed GCTT, is relevant given its potential prognostic and therapeutic implications.<sup>18,24</sup> In metastatic sites, certain histologic patterns of YSTpt (such as glandular/alveolar YSTpt) may be difficult to distinguish from Tpt, which does not require treatment with chemotherapy if there are no concurrent non-teratomatous components.<sup>24</sup> YSTpt may comprise a wide range of histologic patterns, which are often found in combination within individual tumors.<sup>18,19</sup> Given its protean morphologic features, identification of YSTpt on H&E slides can be challenging, even for dedicated urologic pathologists.<sup>18,19</sup> Therefore, immunohistochemical markers for YSTpt that demonstrate good analytical sensitivity and specificity can be clinically helpful in certain scenarios that require distinguishing YSTpt from histologic mimics.<sup>18,19,25-27</sup> Several YSTpt immuno-markers have been evaluated, including well-known and widely used immunostains such as AFP and GPC3, as well as others that are experimental, adopted in academic sets or used as secondary markers with limited

utility [transcription factor encoded by GATA3 (GATA3), homeobox protein CDX-2 (CDX2), hepatocyte nuclear factor 1 homeobox B (HNF1 $\beta$ ), and SOX17].<sup>18,19,25-31</sup> The latter group of markers has demonstrated discrepant results in prior studies, in part due to: a) the heterogeneity of the tested series (testicular tumors vs. ovarian, testicular and/or mediastinal tumors, YSTpt vs. YSTpt and YST prepubertal-type), b) the use of different antibodies and/or immunohistochemical protocols, c) the use of different criteria for classifying cases as positive/negative.<sup>18,19,25-32</sup> Additionally, immunoreactivity for these markers seems to be partially pattern-dependent (glandular/alveolar and hepatoid patterns uncommonly stain for GATA3; solid pattern may exhibit a weak stain for GPC3 and be completely negative for AFP; sarcomatoid pattern is usually positive only for GPC3), and prior studies have not properly evaluated the diagnostic performance of the antibodies in a pattern-specific fashion.<sup>18,19,25-36</sup> In recent years, several authors have focused on the complex mechanisms that drive the transition from seminoma into the other type II GCTT (the so-called “reprogramming of seminoma cells”).<sup>13-16</sup> *Nettersheim et al.*, adopting a combined approach on cell cultures [polymerase chain reaction (PCR), DNA methylation and miRNA analyses, affymetrix expression arrays, and CRISPR/Cas9-mediated genome-editing], identified FOXA2 as a putative driver of YST differentiation, which induces a gene expression profile characteristic of YST phenotype.<sup>13-16</sup> *Wruck W et al.* analyzed 342 histologic samples of GCTT and found that 100% (117/117), 93% (109/117), and 95% (111/117) cases of adult YST (putatively YSTpt) pt were positive for FOXA2, GPC3, and AFP, respectively.<sup>16</sup> However, the authors did not specify the extent and intensity of staining in the individual cases, detail the immunohistochemical criteria used for classifying cases as positive/negative, or assess expression in the different histologic patterns of YSTpt.<sup>16</sup> Notably, the same authors found that 3/3 (100%) pediatric YST cases (putatively YST prepubertal-type) were positive for FOXA2; unfortunately, we did not have YST prepubertal-type cases in our case series. Recently, *Fichtner A et al.* analyzed with immunohistochemistry a cohort of PMGCT and found that all cases of YST [4/4 (100%), 1 pure YST with microcystic/reticular and solid patterns, and 3 mixed YST with microcystic/reticular and endodermal sinus/perivascular

patterns] were positive for AFP, GPC3, and FOXA2.<sup>17</sup> As in the study mentioned above, criteria for classifying cases as positive/negative and evaluation of extent and intensity of staining were not included.<sup>17</sup> The results of these two studies suggested that FOXA2 could represent a useful immunohistochemical marker for testicular YSTpt. In this study, we evaluated the staining pattern of FOXA2 in the different histologic patterns of YSTpt and other GCTT components, comparing this new marker (percentage of positive cells and intensity) with AFP and GPC3. These results are crucial for proposing this marker in the diagnostic routine and planning future studies aimed to analyze its expression in even rarer entities (sarcomatoid YSTpt, YST prepubertal-type, vasculogenic mesenchymal tumor, etc.). In line with prior studies, we found that GPC3 was consistently positive in YSTpt, with a higher sensitivity than AFP.<sup>17-19,23,25-27</sup> GPC3 showed a higher percentage of positive cells and staining intensity higher than AFP in almost all histologic patterns of YSTpt, except for the endodermal sinus/perivascular pattern. Consistent with prior findings, our study demonstrated that the overall performance of GPC3 (both percentage of positive cells and intensity) was better in the most common histologic patterns of YST (microcystic/reticular and myxoid) compared to rarer ones (glandular/alveolar, endodermal sinus/perivascular, solid and polyvesicular vitelline).<sup>18,19,23,25</sup> We found that FOXA2 was positive in all YSTpt, with better performance than GPC3 in terms of percentage of positive cells and intensity mv. FOXA2 showed a higher extent and intensity of staining than GPC3 in almost all histologic patterns of YSTpt (except for macrocystic and polyembryoma/embryoid body), and its performance was superior in rare and difficult-to-diagnose histologic patterns of YSTpt (glandular/alveolar, endodermal sinus/perivascular, solid, and polyvesicular vitelline). Notably, we showed that YSTpt often occurs as a component of mixed GCTT (in particular in combination with EC), and FOXA2 is useful (and better performing than AFP and GPC3) to detect even small foci and/or single cells of YSTpt (*Figure 1E-1H*). This finding suggests that FOXA2 may be useful for the characterization of GCTT with elevated serum AFP levels but no evidence of YSTpt at H&E examination (especially in patients with pure S and elevated serum AFP levels, *bona fide* treated as NSGCTT).<sup>16-19,24</sup> FOXA2

showed higher specificity than AFP and GPC3, being less positive (lower percentage of positive cells and intensity mv) of these two markers in the other GCTT components. Besides, FOXA2 stained only Tpt, thus being less promiscuous than AFP and GPC3, which stained also the other GCTT components (S, EC, etc.). Furthermore, in the subgroup of Tpt, FOXA2 showed intensity mv higher than GPC3 and AFP and a percentage of positive cells higher than AFP (*Table 3* and *Figure 3*). In line with our results, also *Fichtner A et al.* found that FOXA2 was positive only in Tpt (in the group of other GCTT components) with the expression almost exclusively confined to the mature gastrointestinal/respiratory tract epithelium.<sup>17</sup> This data suggests caution in adopting FOXA2 for the differential diagnosis between glandular/alveolar pattern of YSTpt and mature glandular components of Tpt, especially in metastatic sites where Tpt does not require chemotherapy if there are no concurrent non-teratomatous components. Additionally, we did not find GCNIS cases positive for FOXA2 (as well as for AFP and GPC3), again highlighting that FOXA2 is involved in a later phase of the GCTT development.<sup>16</sup> We did not test FOXA2 in other (non-GCTT) tumors (prostate adenocarcinoma, breast carcinoma, melanoma, etc.). Given the reported FOXA2 positivity in specific tumors, we suggest that FOXA2 should be always adopted in combination with SALL4 in the scenario of metastatic disease diagnosis.<sup>1-10</sup> Finally, FOXA2 is a nuclear stain that demonstrated no background reactivity, unlike GPC3 (focal background reactivity in necrotic areas) and AFP (diffuse background reactivity in hemorrhagic and/or necrotic areas, and cystic-glandular fluid secretion). Therefore, FOXA2 may be easier to interpret than AFP and GPC3 in clinical practice.

In conclusion, the present study suggests that FOXA2 is useful for the diagnosis of YSTpt. FOXA2 shows higher sensitivity and specificity than AFP and GPC3, with no background reactivity. Our results suggest that FOXA2 may be particularly useful to identify rare YSTpt histologic patterns that are difficult to diagnose, but that mature glandular components of Tpt could represent a potential diagnostic pitfall. Further studies are needed to evaluate FOXA2 in histologic patterns of



YSTpt not analyzed in this study (sarcomatoid YSTpt and vasculogenic mesenchymal tumor) and other neoplasms that could be included in the differential diagnosis of YSTpt (especially in metastatic sites).

## References

1. Jimenez FR, Lewis JB, Belgique ST, et al. Developmental lung expression and transcriptional regulation of claudin-6 by TTF-1, Gata-6, and FoxA2. *Respir Res.* 2014;15(1):70.
2. Cho JW, Lee CY, Ko Y. Therapeutic potential of mesenchymal stem cells overexpressing human forkhead box A2 gene in the regeneration of damaged liver tissues. *J Gastroenterol Hepatol.* 2012;27(8):1362-70.
3. Howard L, Mackenzie RM, Pchelintsev NA, et al. Profiling of transcriptional and epigenetic changes during directed endothelial differentiation of human embryonic stem cells identifies FOXA2 as a marker of early mesoderm commitment. *Stem Cell Res Ther.* 2013;4(2):36.
4. Tang Y, Shu G, Yuan X, et al. FOXA2 functions as a suppressor of tumor metastasis by inhibition of epithelial-to-mesenchymal transition in human lung cancers. *Cell Res.* 2011;21(2):316-26.
5. Song Y, Washington MK, Crawford HC. Loss of FOXA1/2 is essential for the epithelial-to-mesenchymal transition in pancreatic cancer. *Cancer Res.* 2010;70(5):2115-25.
6. Han M, Li F, Zhang Y, et al. FOXA2 drives lineage plasticity and KIT pathway activation in neuroendocrine prostate cancer. *Cancer Cell.* 2022;40(11):1306-23.
7. Basseres DS, D'Alò F, Yeap BY, et al. Frequent downregulation of the transcription factor Foxa2 in lung cancer through epigenetic silencing. *Lung Cancer.* 2012;77(1):31-7.
8. Lin J, Zhang D, Fan Y, et al. Regulation of Cancer Stem Cell Self-Renewal by HOXB9 Antagonizes Endoplasmic Reticulum Stress-Induced Melanoma Cell Apoptosis via the miR-765-FOXA2 Axis. *J Invest Dermatol.* 2018;138(7):1609-19.
9. Shang H, Shi L, Jiang X, et al. Correlation Between High Expression of FOXA2 and Improved Overall Survival in Ovarian Cancer Patients. *Med Sci Monit.* 2021;27:e928763.

10. Gao H, Yan Z, Sun H, et al. FOXA2 promotes esophageal squamous cell carcinoma progression by ZEB2 activation. *World J Surg Oncol*. 2021;19(1):286.
11. Aghadi M, Elgendy R, Abdelalim EM. Loss of FOXA2 induces ER stress and hepatic steatosis and alters developmental gene expression in human iPSC-derived hepatocytes. *Cell Death Dis*. 2022;13(8):713.
12. Choi W, Yang AX, Sieve A, et al. Pulmonary Mycosis Drives Forkhead Box Protein A2 Degradation and Mucus Hypersecretion through Activation of the Spleen Tyrosine Kinase-Epidermal Growth Factor Receptor-AKT/Extracellular Signal-Regulated Kinase 1/2 Signaling. *Am J Pathol*. 2021;191(1):108-30.
13. Nettersheim D, Heimsoeth A, Jostes S, et al. SOX2 is essential for in vivo reprogramming of seminoma-like TCam-2 cells to an embryonal carcinoma-like fate. *Oncotarget*. 2016;7(30):47095-110.
14. Nettersheim D, Schorle H. The plasticity of germ cell cancers and its dependence on the cellular microenvironment. *J Cell Mol Med*. 2017;21(8):1463-7.
15. Nettersheim D, Vadder S, Jostes S, et al. TCam-2 Cells Deficient for SOX2 and FOXA2 Are Blocked in Differentiation and Maintain a Seminoma-Like Cell Fate In Vivo. *Cancers (Basel)*. 2019;11(5):728.
16. Wruck W, Bremmer F, Kotthoff M, et al. The pioneer and differentiation factor FOXA2 is a key driver of yolk-sac tumour formation and a new biomarker for paediatric and adult yolk-sac tumours. *J Cell Mol Med*. 2021;25(3):1394-405.
17. Fichtner A, Richter A, Filmar S, et al. Primary mediastinal germ cell tumours: an immunohistochemical and molecular diagnostic approach. *Histopathology*. 2022 Jan;80(2):381-96.
18. WHO Classifications of Tumours Editorial Board. Urinary and Male Genital Tumours. IARC Press. Lyon. 2022 (WHO classification of tumours series, 5<sup>th</sup> ed.; vol.8).

19. Ulbright TM. Pitfalls in the interpretation of specimens from patients with testicular tumours, with an emphasis on variant morphologies. *Pathology*. 2018;50(1):88-99.
20. Ricci C, Ambrosi F, Franceschini T, et al. Yolk sac tumor of postpubertal-type does not exhibit immunohistochemical loss of SMARCB1/INI1 and SMARCA4/BRG1...but choriocarcinoma? *Pathol Res Pract*. 2022;241:154269.
21. Ricci C, Franceschini T, Giunchi F, et al. Immunohistochemical Expression of Preferentially Expressed Antigen in Melanoma (PRAME) in the Uninvolved Background Testis, Germ Cell Neoplasia In Situ, and Germ Cell Tumors of the Testis. *Am J Clin Pathol*. 2022;157(5):644-8.
22. Orsatti A, Sirolli M, Ambrosi F, et al. SOX2 and PRAME in the "reprogramming" of seminoma cells. *Pathol Res Pract*. 2022;237:154044.
23. Zynger DL, McCallum JC, Luan C, et al. Glypican 3 has a higher sensitivity than alpha-fetoprotein for testicular and ovarian yolk sac tumour: immunohistochemical investigation with analysis of histological growth patterns. *Histopathology*. 2010;56(6):750-7.
24. Gilligan T, Lin DW, Aggarwal R, et al. Testicular Cancer, Version 2.2020, NCCN Clinical Practice Guidelines in Oncology. *J Natl Compr Canc Netw*. 2019;17(12):1529-54.
25. Siegmund SE, Mehra R, Acosta AM. An update on diagnostic tissue-based biomarkers in testicular tumors. *Hum Pathol*. 2022 Aug 3:S0046-8177(22)00204-0. doi: 10.1016/j.humpath.2022.07.020. *Online ahead of print*.
26. Emerson RE, Ulbright TM. The use of immunohistochemistry in the differential diagnosis of tumors of the testis and paratestis. *Semin Diagn Pathol*. 2005;22(1):33-50.
27. Ulbright TM, Tickoo SK, Berney DM, et al. Best practices recommendations in the application of immunohistochemistry in testicular tumors: report from the International Society of Urological Pathology consensus conference. *Am J Surg Pathol*. 2014;38(8):e50-9.

28. Schuldt M, Rubio A, Preda O, et al. GATA binding protein 3 expression is present in primitive patterns of yolk sac tumours but is not expressed by differentiated variants. *Histopathology*. 2016;68(4):613-5.
29. Osman H, Cheng L, Ulbright TM, et al. The utility of CDX2, GATA3, and DOG1 in the diagnosis of testicular neoplasms: an immunohistochemical study of 109 cases. *Hum Pathol*. 2016;48:18-24.
30. Rougemont AL, Tille JC. Role of HNF1 $\beta$  in the differential diagnosis of yolk sac tumor from other germ cell tumors. *Hum Pathol*. 2018;81:26-36.
31. Nonaka D. Differential expression of SOX2 and SOX17 in testicular germ cell tumors. *Am J Clin Pathol*. 2009;131(5):731-6.
32. Gallo A, Fankhauser C, Hermanns T, et al. HNF1 $\beta$  is a sensitive and specific novel marker for yolk sac tumor: a tissue microarray analysis of 601 testicular germ cell tumors. *Mod Pathol*. 2020;33(11):2354-60.
33. Kao CS, Idrees MT, Young RH, et al. Solid pattern yolk sac tumor: a morphologic and immunohistochemical study of 52 cases. *Am J Surg Pathol*. 2012;36(3):360-7.
34. Al-Obaidy KI, Williamson SR, Shelman N, et al. Hepatoid Teratoma, Hepatoid Yolk Sac Tumor, and Hepatocellular Carcinoma: A Morphologic and Immunohistochemical Study of 30 Cases. *Am J Surg Pathol*. 2021;45(1):127-36.
35. Young RH, Ulbright TM, Policarpio-Nicolas MLC. Yolk sac tumor with a prominent polyvesicular vitelline pattern: a report of three cases. *Am J Surg Pathol*. 2013;37(3):393-8.
36. Howitt BE, Magers MJ, Rice KR, et al. Many postchemotherapy sarcomatous tumors in patients with testicular germ cell tumors are sarcomatoid yolk sac tumors: a study of 33 cases. *Am J Surg Pathol*. 2015;39(2):251-9.

## Figure Legends.

### Figure 1.

forkhead box transcription factor A2 (FOXA2), glypican-3 (GPC3),  $\alpha$ -fetoprotein (AFP), yolk sac tumor postpubertal-type (YSTpt).

#### **FOXA2, GPC3, and AFP in the different histologic patterns of YSTpt.**

Microcystic/reticular pattern of YSTpt (A: H&E, B: FOXA2, C: GPC3, D: AFP; original magnification 80x) clearly stained by FOXA2 and GPC3, but showing a focal and weak positivity for AFP. Small foci of microcystic/reticular pattern of YSTpt (E: H&E, F: FOXA2, G: GPC3, H: AFP; original magnification 80x) highlighted by FOXA2, but not by GPC3 and AFP.

Solid and macrocystic patterns of YSTpt (I: H&E, J: FOXA2, K: GPC3, L: AFP; original magnification 80x) exhibiting diffuse and strong stain with FOXA2 and GPC3, but only focal stain for AFP. Solid and microcystic/reticular patterns of YSTpt (M: H&E, N: FOXA2, O: GPC3, P: AFP; original magnification 80x) with diffuse and strong stain for FOXA2 and GPC3 in both patterns, but only focal stain for AFP in microcystic/reticular pattern.

Note the diffuse background reactivity of AFP, the focal background reactivity of GPC3 in necrotic areas and cystic-glandular fluid secretions, and the complete absence of background reactivity with an easily interpretable nuclear signal of FOXA2.

### Figure 2.

forkhead box transcription factor A2 (FOXA2), glypican-3 (GPC3),  $\alpha$ -fetoprotein (AFP), yolk sac tumor postpubertal-type (YSTpt).

#### **FOXA2, GPC3, and AFP in the different histologic patterns of YSTpt.**

Polyembryoma/embryoid body pattern of YSTpt (A: H&E, B: FOXA2, C: GPC3, D: AFP; original magnification 200x) with the dorsal amnion-like cavity of the embryoid body exhibiting strong and diffuse stain for FOXA2 and GPC3, and moderate stain for AFP. Unfortunately, the image slightly changed in the subsequent sections for immunohistochemistry. Microcystic/reticular (black star), glandular/alveolar (black triangle), and polyvesicular vitelline (black arrow) patterns of YSTpt (E: H&E, F: FOXA2, G: GPC3, H: AFP; original magnification 200x). FOXA2 shows strong stain in microcystic/reticular and glandular/alveolar patterns, but only weak stain in polyvesicular vitelline pattern. In contrast, GPC3 was positive only in microcystic/reticular pattern, and AFP was completely negative in all patterns.

### **Figure 3.**

forkhead box transcription factor A2 (FOXA2), glypican-3 (GPC3),  $\alpha$ -fetoprotein (AFP), choriocarcinoma (CHC), embryonal carcinoma (EC), teratoma postpubertal-type (Tpt).

### **FOXA2, GPC3, and AFP in the different histologic patterns of YSTpt.**

CHC (A: H&E, B: FOXA2, C: GPC3, D: AFP; original magnification 100x). GPC3 showed strong and diffuse expression in syncytiotrophoblast cells, but only focal expression in cytotrophoblast and intermediate trophoblast cells. AFP showed focal and weak stain in syncytiotrophoblast cells. EC (E: H&E, F: FOXA2, G: GPC3, H: AFP; original magnification 100x) with focal and weak GPC3 expression in the epithelium, and completely negative for FOXA2 and AFP. Tpt (I: H&E, J: FOXA2, K: GPC3, L: AFP; original magnification 100x) showed strong FOXA2 expression in some mature glands. Note the background reactivity of AFP in stroma and cystic-glandular fluid secretions.