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Author contributions

-All authors contributed to the study conception and design.

-Material preparation, data collection and analysis were performed by Costantino Ricci, Francesca Ambrosi, Alessia Grillini, and Andres Martin Acosta;

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Abstract

In the post-chemotherapy setting, germ cell tumors of the testis (GCTT) that resemble non-specific sarcomas and co-express cytokeratins and glypican-3 (GPC3) are diagnosed as "sarcomatoid yolk sac tumor postpubertal-type (YSTpt)". The diagnosis of sarcomatoid YSTpt is clinically relevant but challenging due to its rarity, non-specific histology, and negative α-fetoprotein (AFP) staining. Recently, *FOXA2* has emerged as a key-gene in the reprogramming of GCTT (activating the transcription of several genes, among which *GATA3*), and immunohistochemical studies showed that GATA3 and FOXA2 have a higher sensitivity for non-sarcomatoid YSTpt than GPC3 and AFP. We found that sarcomatoid YSTpt did not express FOXA2 [0: 14/14 (100%)] and showed focal expression of GATA3 [0: 12/14 (85.7%), 1+: 2/14 (14.3%)], thus suggesting that these markers are not useful in diagnosing this tumor. Furthermore, we proposed a potential mechanism of sarcomatoid transformation in the post-chemotherapy setting of GCTT, mediated by the downregulation of *FOXA2* and *GATA3*.

Keywords: GATA3, FOXA2, yolk sac tumor postpubertal-type, sarcomatoid yolk sac tumor postpubertal-type, somatic-type malignancy.

Introduction

The rare occurrence of overgrowth of histologic components resembling somatic malignancies (STMs) in germ cell tumors of the testis (GCTT) is usually observed after chemotherapy and at metastatic sites [1-7]. STMs are chemoresistant and characterized by multiple recurrences and poor disease-specific survival; histologically, STMs span from embryonic-type neuroectodermal tumor to nephroblastoma, STMs with mature neuroglial phenotype (potentially resembling all central nervous system tumors), carcinomas, and sarcomas [1-7]. The latter may show a somewhat broad phenotypic spectrum, including tumors that closely resemble well-defined types of true somatic sarcomas (e.g., rhabdomyosarcoma, chondrosarcoma) at one end, and undifferentiated spindle cell sarcomas at the other [1-7]. Historically, STMs of germ cell origin were thought to arise in teratoma postpubertal-type (Tpt), but some authors have previously suggested that a relatively significant subset of STMs occurring in the post-chemotherapy setting (as late or very late recurrences) arise in yolk sac tumor postpubertal-type (YSTpt) [2, 3, 7]. *Bremmer F et al* recently found, adopting complex DNA methylation and proteomic data analyses, that carcinomatous STMs resemble YSTpt and sarcomatous STMs resemble Tpt [7]. In the clinical scenario of post-chemotherapy relapse of GCTT, a poorly described subset of spindle and/or epithelioid cell neoplasms with fibromyxoid stroma expressing cytokeratins, glypican-3 (GPC3) and SALL4 is considered sarcomatoid YSTpt rather than a specific subtype of sarcomatous STMs [1, 2, 8]. The appropriate identification of sarcomatoid YSTpt is clinically relevant due to its aggressive biological features comparable to those of sarcomatous STMs (chemoresistance, high frequency of recurrence, and poor disease-specific outcome). However, its diagnosis is challenging due to its rarity, unremarkable histological features (mixture of spindle and epithelioid cells, fibromyxoid stroma with delicate vascular network of capillaries, absence or small residual component of “classic” histologic patterns of YSTpt), and frequent negative or low expression of commonly used stains (AFP is negative, SALL4 is focally/moderately positive in about 60% of cases, and the threshold used to define positive cytokeratins and GPC3 and to diagnose YSTpt, is “only” 10% of stained cells) [1, 2, 8]. In

recent years, data from developmental biology studies have identified the forkhead box transcription factor A2 (*FOXA2*) as an early determinant of the transition from embryonal carcinoma to YSTpt (i.e., reprogramming), controlling gene expression programs that activate transcription of genes typically associated with a mature YSTpt phenotype [*GPC3*, α-fetoprotein (*AFP*), *GATA3*] [9, 10]. Subsequent immunohistochemical studies suggested that GATA3 and FOXA2 may have a higher sensitivity for YSTpt than classical markers such as GPC3 and AFP, and thus may be relevant tools for the diagnosis of YSTpt [9, 11]. Specifically, the sensitivity of FOXA2 approached 100% for non-sarcomatoid YSTpt patterns, while data on GATA3 were discrepant, with sensitivity ranging from 12% to 100% for non-sarcomatoid YSTpt patterns and previous authors found correspondence between the staining pattern and the histologic patterns [GATA3 preferentially expressed in the so-called "primitive patterns" (microcystic/reticular, endodermal sinus/perivascular, polyvesicular vitelline, primitive structures polyembryoma/embryoid body) and negative in the "differentiated patterns" (glandular/alveolar and hepatoid)] [9-13]. However, although FOXA2 and (to some extent) GATA3 are thought to initiate and regulate the induction of the YSTpt phenotype, their immunohistochemical expression has never been investigated in sarcomatoid YSTpt [9-13]. To the best of our knowledge, we report here the first case series of sarcomatoid YSTpt immunohistochemically assessed for FOXA2 and GATA3.

Case series

We collected 14 cases (11 patients) of sarcomatoid YSTpt diagnosed at the Department of Pathology of Indiana University. All cases in this cohort have been previously published by the Indiana group but were reviewed (A.M.A. and T.M.U) to confirm the original diagnosis of sarcomatoid YSTpt according to the histologic and immunohistochemical criteria (AFP negative, GPC3 and cytokeratins with ≥10% of tumor cells showing at least moderately intense reactivity)

and select a representative block for immunohistochemical analysis [2, 8]. Two consecutive 3-µm sections were cut from each selected paraffin-embedded tissue block and stained with GATA3 and FOXA2 (BenchMark ULTRA automated immunostainer; Ventana Medical Systems-Roche Diagnostics, Basel, Switzerland), as previously described [11]. Standardized in-house and commercial positive control tissues were adopted for GATA3 (breast tissue) and FOXA2 (colorectal mucosa) [11]. Slides stained for GATA3 and FOXA2 (both with nuclear staining) were read simultaneously by two dedicated uropathologists (M.F. and C.R.) on a multihead microscope and expression was assessed as previously described [percentage: 0 (<5%), 1+ (5-10%), 2+ (11-50%), 3+ (>50%); intensity: 0 (no staining), 1 (weak), 2 (moderate), 3 (strong)] [2]. The study has been approved by the Institutional Review Board (IRB) of Indiana University (Protocol #18697, 2023). All cases were negative for FOXA2 [percentage and intensity: 0 in 14/14 (100%) cases]. A small subset of cases showed focal expression of GATA3 [0: 12/14 (85.7%), 1+: 2/14 (14.3%)], with 2 cases showing scattered positive cells (<5%) but below the established cut-off for 1+ score; staining was weak in all GATA3-positive tumors [1: 4/14 (28.6%); considering the two 1+ cases and the 2 cases with <5% of positive cells] (*Figure 1* and *Table 1*). According to the common overwhelming aspect of sarcomatoid YSTpt, no other GCTT components and/or non-sarcomatoid YSTpt patterns were detected in the selected slides.

Discussion

In the clinical scenario of post-chemotherapy recurrences of GCTT, the diagnosis of sarcomatoid YSTpt is challenging but clinically relevant, due to its chemoresistance, tendency to recur and poor prognosis [1, 2, 8]. Two promising markers for the development and diagnosis of YSTpt, namely GATA3 and FOXA2, have never been analyzed in sarcomatoid YSTpt [1, 2, 8-13]. GATA3 is a transcription factor that controls the expression of numerous genes involved in crucial steps of embryonic development (neural crest cells, lymphocytes, mammary glands, etc.), as well as in the

biology of various tumors (immune response, metastasis, specific lineage differentiation, etc.) [14]. Initially, GATA3 was found to be highly specific for breast and urothelial tumors, but it was subsequently found to be expressed in a wide variety of urological (choriocarcinoma and other trophoblastic tumors of the testis, low-grade oncocytic tumor of the kidney, clear cell renal cell tumor, etc.) and non-urological (paraganglioma and pheochromocytoma, salivary gland neoplasms, mesothelioma, etc.) tumors [14]. The present study shows that GATA3 and FOXA2 are not useful markers for the diagnosis of sarcomatoid YSTpt, whose diagnosis is still based on clinical data, morphologic assessment, and immunohistochemical markers commonly adopted for non-sarcomatoid YSTpt (SALL4, AFP, and GPC3) [1, 2, 8]. However, our results are hypothesis-generating and suggest possible mechanisms of sarcomatoid transformation in the post-chemotherapy setting of these tumors. Specifically, the transition from non-sarcomatoid YSTpt to sarcomatoid YSTpt may be mediated in part by downregulation of *FOXA2* and, subsequently, *GATA3*, leading to loss of the typical YSTpt phenotype (i.e., "dedifferentiation") [9]. Based on previous studies in other tumor types, the downregulation of *FOXA2* is most likely driven by epigenetic mechanisms, and it is strongly implicated in the epithelial-mesenchymal transition, thus potentially prompting the histologic and immunohistochemical sarcoma-like phenotype of this entity [15, 16]. Subsequently, additional genomic lesions (e.g., events leading to *TP53* inactivation) may promote further biological progression, including transformation to high-grade unclassified pleomorphic sarcomas of germ cell origin (*Figure 2*) [8]. Otherwise, negativity for GATA3 and FOXA2 could raise the suspicion that sarcomatoid YSTpt is not a histologic pattern of YSTpt but rather a specific type of sarcomatous STM. Although the distinction between these two entities is largely semantic (clinical, prognostic, and therapeutic implications do not appear to be significantly different), we agree with other authors that the amount of knowledge still supports the “YSTpt nature” of sarcomatoid YSTpt (histologic appearance suggesting a progression of myxoid and microcystic/reticular patterns, rare association with non-sarcomatoid YSTpt patterns, expression of SALL4, GPC3, and cytokeratins) and differentiation from other sarcomatous STMs until their

pathogenesis is clearly elucidated (potentially allowing the adoption of targeted therapy for specific molecular biological pathways) [2].

Figure Legends.

Fig. 1. FOXA2 and GATA3 in sarcomatoid YSTpt.

yolk sac tumor postpubertal-type (YSTpt);

Sarcomatoid YSTpt showing myxoid stroma, predominantly spindle cells, and reticular architecture with a delicate vascular network of capillaries [A: H&E, original magnification x130], focally positive for GATA3 (1+) [B, original magnification x130] but completely negative for FOXA2 (0) [C, original magnification x130].

Sarcomatoid YSTpt showing fibrotic stroma, predominantly spindle cells, and vague resemblance of reticular architecture [D: H&E, original magnification x130], completely negative for GATA3 (0) [E, original magnification x130] and FOXA2 (0) [F, original magnification x130]. GATA3 stain scattered T-lymphocytes (positive internal control).

Small foci of microcystic/reticular YSTpt intermingled with embryonal carcinoma [G: H&E, original magnification x130], and resulted diffusely positive for GATA3 (percentage: 3+; intensity: 2) [H, original magnification x130] and FOXA2 (percentage: 3+; intensity: 3) [I, original magnification x130]. Note that the embryonal carcinoma is completely negative (0) for both stains.

Fig. 2. Possible mechanism for transition from non-sarcomatoid YSTpt to sarcomatoid YSTpt and further biological progression.

yolk sac tumor postpubertal-type (YSTpt);

Green arrows: reduced expression; red arrows: increased expression;

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Declarations

1) Funding and Competing interests

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2) Ethics approval, Consent, Data, Material and/or Code availability

Study-specific approval by the appropriate ethics committee for research involving humans and/or animals: The study has been approved by the Institutional Review Board (IRB) of Indiana University (Protocol #18697, 2023);

Informed consent if the research involved human participants: All patients included in the study provided informed consent, after a consultation with the investigators;

Data, Material, and/or Code availability: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request;

3) Authors' contribution statements

-All authors contributed to the study conception and design.

-Material preparation, data collection and analysis were performed by Costantino Ricci, Francesca Ambrosi, Alessia Grillini, and Andres Martin Acosta;

-Writing - original draft preparation: Costantino Ricci, Michelangelo Fiorentino, Maurizio Colecchia, Francesco Massari, Thomas M Ulbright, and Andres Martin Acosta;

-Writing - review and editing: Costantino Ricci, Maurizio Colecchia, Andres Martin Acosta, and Thomas M Ulbright;

-All authors read and approved the final manuscript.

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