Human Pathology (2019) **84**, 254–261

ELSEVIER

Human PATHOLOGY

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Original contribution

Cytokeratin 7, inhibin, and p63 in testicular germ cell tumor: superior markers of choriocarcinoma compared to β-human chorionic gonadotropin☆

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Received 22 August 2018; revised 2 October 2018; accepted 11 October 2018

Keywords:
Testicle;
Germ cell tumor;
Choriocarcinoma;
CK7;
Inhibin;
p63;
β-hCG

Summary Choriocarcinoma can be difficult to differentiate from other subtypes of testicular germ cell tumor and can occur unexpectedly in a distant, late metastasis. The aim of this investigation was to identify a marker superior to β-human chorionic gonadotropin (β-hCG) for choriocarcinoma. Sixty-two primary and metastatic testicular germ cell tumors (27 choriocarcinomas, 19 yolk sac tumors, 29 embryonal carcinomas, 28 seminomas, 22 teratomas, 3 epithelioid trophoblastic tumors [ETTs]) were analyzed for immunohistochemical expression of cytokeratin 7 (CK7), inhibin, p63, and β-hCG. All choriocarcinomas and ETTs were strongly positive for CK7, whereas seminomas were negative and 52% of embryonal carcinomas had weak reactivity. Eighty-four percent of yolk sac tumors and 59% of teratomas were CK7 positive. Eighty-nine percent of choriocarcinomas and 100% of ETTs were positive for inhibin, with reactivity highlighting syncytiotrophoblasts, whereas seminomas, embryonal carcinomas, yolk sac tumors, and teratomas were negative. Eighty-five percent of choriocarcinomas expressed p63, with staining mostly in mononucleated trophoblasts, whereas seminomas, embryonal carcinomas, and yolk sac tumors were negative. Teratomas expressed p63 in 32% of cases. β-hCG was reactive in 96% of choriocarcinomas, 33% of ETTs, 46% of seminomas, 54% of embryonal carcinomas, 47% of yolk sac tumors, and 32% of teratomas. β-hCG staining within other subtypes was more likely if choriocarcinoma was present elsewhere in the tumor ($P = .0002$). CK7 is a highly sensitive marker for choriocarcinoma and differentiates choriocarcinoma from seminoma and embryonal carcinoma. Inhibin and p63 are sensitive and specific for choriocarcinoma versus seminoma, embryonal carcinoma, and yolk sac tumor. To identify choriocarcinoma, CK7, inhibin, and p63 are superior to β-hCG.

1. Introduction

The most common type of testicular malignancy is testicular germ cell tumor (GCT). The subtypes of testicular GCT may exhibit morphologic overlap making accurate histologic diagnosis difficult, particularly in the setting of mixed GCT. To make a histologic diagnosis, it is important to accurately identify and quantify the subtypes that are present within the

☆ Funding/Support: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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https://doi.org/10.1016/j.humpath.2018.10.007
0046-8177/

tumor, as this can affect prognosis and management [1]. Specifically, choriocarcinoma can be difficult to diagnose as other subtypes, namely, seminoma and embryonal carcinoma, may mimic the histologic appearance of choriocarcinoma [2,3]. Choriocarcinoma is one of the more aggressive subtypes of GCT, and therefore, accurate identification is vital. The subtypes of GCT can also display overlap of immunohistochemical expression, and thus far, a sensitive and specific marker for testicular choriocarcinoma has not been described in the literature. The most widely studied and used immunohistochemical marker for choriocarcinoma is β-human chorionic gonadotropin (β-hCG), but this marker lacks specificity for choriocarcinoma and often displays extensive background staining [2].

The aim of our study was to compare the differential expression of several widely available immunohistochemical markers that have been poorly studied in testicular choriocarcinoma, including cytokeratin 7 (CK7), inhibin, and p63, to β-hCG in order to identify a superior marker.

2. Materials and methods

2.1. Case selection

Archived, formalin-fixed, paraffin-embedded tissue blocks from 62 cases of primary or metastatic testicular GCT accessioned between 2000 and 2017 were obtained from the surgical pathology files of The Ohio State University Wexner Medical Center. Serum β-hCG before orchiectomy was recorded. The 62 cases included 50 testicular primary tumors and 12 metastases (2 central nervous system, 3 lung, 4 lymph node, 1 chest, 1 shoulder, 1 pleura). The 50 cases of testicular primary tumor consisted of 35 mixed GCTs, 13 pure seminomas, and 2 pure choriocarcinomas. The 12 cases of metastatic GCT included 6 choriocarcinomas, 3 mixed GCTs, and 3 epithelioid trophoblastic tumors (ETTs). In aggregate, the 62 cases of GCT included the following histologic subtypes: 27 choriocarcinomas, 29 embryonal carcinomas, 19 yolk sac tumors, 22 teratomas, 28 seminomas, and 3 ETTs. Two cases of seminoma contained syncytiotrophoblastic cells. The syncytiotrophoblastic component when present was scored separately from the other seminoma cells. Syncytiotrophoblasts were not present in any other nonchoriocarcinoma tumor subtype. This study was approved by The Ohio State University Cancer Institutional Review Board and was performed in compliance with our institutional review board guidelines (study number 2016C0168).

2.2. Immunohistochemistry

Sections (5 μm) from one representative block from each case were deparaffinized, rehydrated in graded alcohols, and subjected to heat-induced epitope retrieval in 0.1 mol/L of citrate buffer at pH 6.0 in a microwave for 20 minutes. The slides were subsequently incubated with a primary monoclonal antibody specific for either CK7 (1:600 dilution, clone OV-TL-12/30; Dako), inhibin (1:60 dilution, clone R1; Dako), p63 (1:300 dilution, clone BC4A4; Biocare Medical), or β-hCG (1:800 dilution, clone R-poly; Dako).

Each histologic subtype was individually analyzed for membranous and cytoplasmic staining of CK7, inhibin, and β-hCG, and nuclear staining of p63. Immunoreactivity was semiquantitatively evaluated as negative (0, <5% of cells stained), focally positive (1+, 5%-10% of cells stained), positive (2+, 11%-50% of cells stained), and diffusely positive (3+, >50% of cells stained). Staining intensity, when present, was semiquantitatively evaluated as weak (1), moderate (2), or strong (3). Appropriate positive and negative controls were used. One case of mixed GCT containing choriocarcinoma did not have choriocarcinoma on the slide stained for p63 and was omitted from analysis of this marker, as additional unstained slides could not be obtained. For cases with β-hCG immunohistochemical positivity but no documented choriocarcinoma or syncytiotrophoblasts, all slides from the case were reexamined to ensure the absence of trace choriocarcinoma or syncytiotrophoblasts elsewhere within the submitted tumor.

2.3. Statistical analysis

A 2-tailed Fisher exact test was performed to evaluate differences in β-hCG reactivity between tumors with choriocarcinoma or syncytiotrophoblasts present in any block of the case and tumors without choriocarcinoma or syncytiotrophoblasts present in any block. A P value of .05 or less was considered significant.

3. Results

3.1. CK7

All choriocarcinomas (8 pure, 19 mixed GCT) were diffusely positive for CK7 (3+, 100%) and exhibited strong or moderate staining intensity (mean, 3.0; Tables 1 and 2, Figs. 1-3). Both syncytiotrophoblasts and mononucleated trophoblasts were equally reactive. Although more than half of

Table 1 Percent positivity of CK7, inhibin, p63, and β-hCG in testicular GCT subtypes

GCT subtype	%			
	CK7	Inhibin	p63	β-hCG
Choriocarcinoma	100	89	85	96
Embryonal carcinoma	52	0	0	55
Yolk sac tumor	84	0	0	47
Teratoma	59	0	32	32
Seminoma	0	0	0	46
ETT	100	100	100	33

Table 2 CK7 immunoreactivity in testicular GCT subtypes

Score (% of cells stained)	n (%)					
	CC	EC	YST	TR	SE	ETT
3+ (>50)	27 (100)	1 (3)	5 (26)	2 (9)	0	3 (100)
2+ (11-50)	0	7 (24)	6 (31)	2 (9)	0	0
1+ (5-10)	0	7 (24)	5 (26)	9 (41)	0	0
0 (<5)	0	14 (48)	3 (16)	9 (41)	28 (100)	0
Total no.	27	29	19	22	28	3
Mean intensity	3.0	1.1	2.5	3.0	–	3.0

Abbreviations: CC, choriocarcinoma; EC, embryonal carcinoma; ETT, epithelial trophoblastic tumor; SE, seminoma; TR, teratoma; YST, yolk sac tumor.

embryonal carcinomas had reactivity to CK7 (52%), the staining pattern was rarely diffuse and the intensity was weak (mean, 1.1) in most cases. Yolk sac tumors were usually positive for CK7 (84%) with strong staining intensity (mean, 2.5) in most cases. Teratomas had variable reactivity to CK7 (59%) that was limited to the epithelial components, but staining intensity was strong when present (mean, 3.0). All seminomas were negative for CK7. In the 2 cases of seminoma containing syncytiotrophoblasts, CK7 was positive (3+, 100%) in all synctiotrophoblastic cells with strong intensity (mean, 2.5). Similar to choriocarcinomas, all ETTs were diffusely (3+, 100%) and strongly positive for CK7 (mean, 3.0).

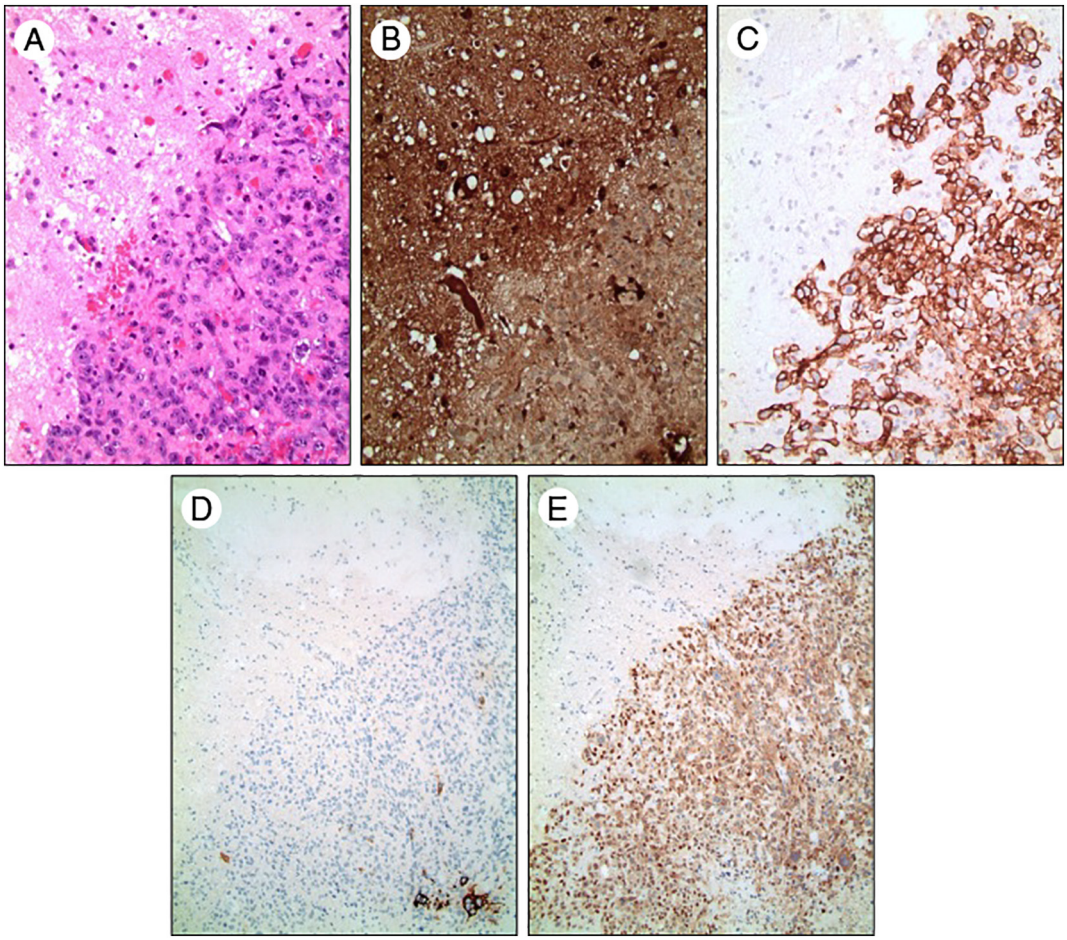

Fig. 1 Choriocarcinoma metastatic to the brain (original magnifications ×20 for all images). β-hCG showed extensive nonspecific staining, which was not observed with CK7, inhibin, or p63. A, Hematoxylin and eosin staining of metastatic choriocarcinoma (lower right) and brain tissue (upper left). B, Diffuse, nonspecific staining of β-hCG in choriocarcinoma and brain tissue. C, Positive CK7 staining in choriocarcinoma and negative in brain tissue. D, Inhibin reactivity in rare syncytiotrophoblasts and negative in brain tissue. E, p63 is positive in mononucleated trophoblasts and negative in brain tissue.

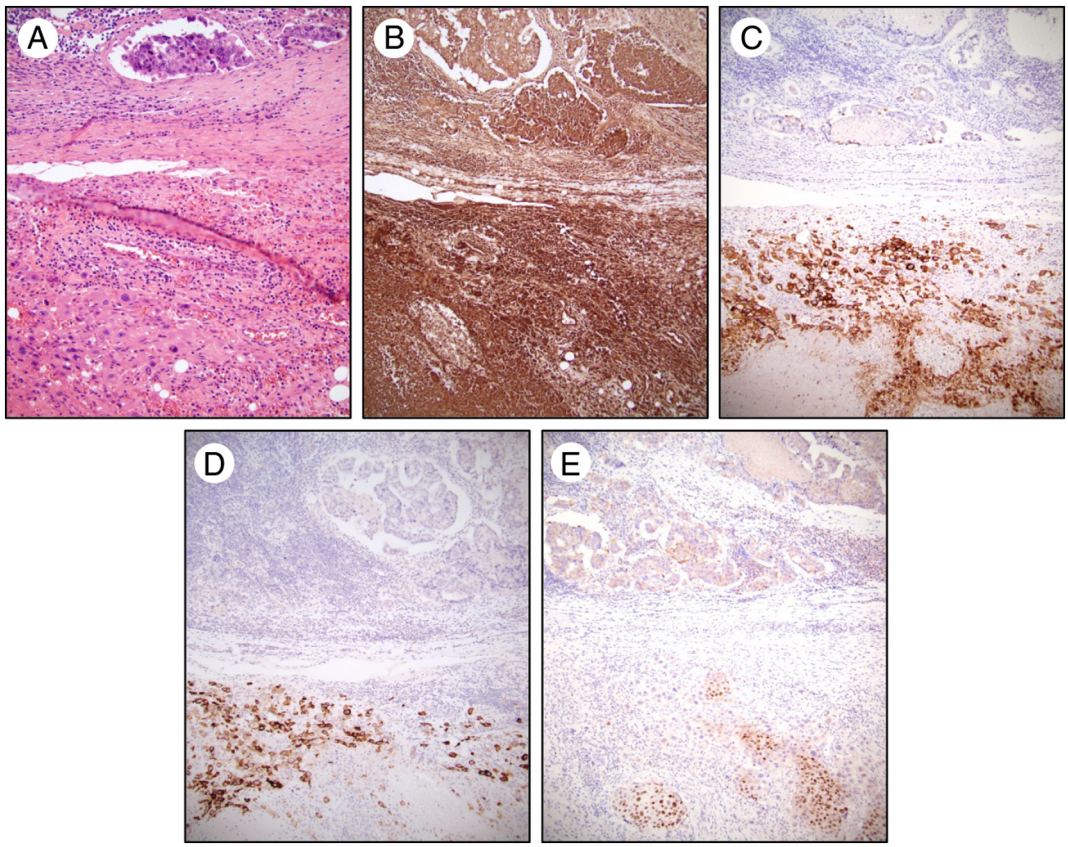

Fig. 2 Choriocarcinoma and embryonal carcinoma metastatic to an axillary lymph node (original magnifications ×10 for all images). Positivity for CK7, inhibin, and p63 differentiated choriocarcinoma from embryonal carcinoma, whereas β-hCG did not. A, Hematoxylin and eosin staining with choriocarcinoma (bottom) and embryonal carcinoma (top). B, Diffuse, nonspecific β-hCG reactivity in both choriocarcinoma and embryonal carcinoma. C, CK7 was positive in choriocarcinoma and negative in embryonal carcinoma. D, Inhibin was positive in choriocarcinoma and negative in embryonal carcinoma. E, p63 was positive in choriocarcinoma and negative in embryonal carcinoma.

3.2. Inhibin

Most choriocarcinomas (89%) were positive for inhibin (3+, 37%; 2+, 41%; 1+, 11%), and most cases displayed strong staining intensity (mean, 2.7; Tables 1 and 3, Figs. 1-3). The syncytiotrophoblastic component of choriocarcinoma demonstrated higher reactivity for inhibin, with relative sparing of the mononucleated trophoblastic cells. Yolk sac tumors, embryonal carcinomas, seminomas, and teratomas were negative for inhibin. The 2 cases of seminoma containing syncytiotrophoblasts were positive for inhibin (1+, 50%; 2+, 50%) with a mean intensity of 1.5. All ETTs were positive for inhibin (3+, 33%; 2+, 33%; 1+, 33%) with strong or moderate intensity (mean, 2.7).

3.3. p63

Most cases of choriocarcinoma (85%) were positive for p63 (3+, 15%; 2+, 50%; 1+, 19%) and exhibited strong or moderate intensity (mean, 2.7; Tables 1 and 4, Figs. 1-3). In contrast to inhibin, the mononucleated trophoblastic component of choriocarcinoma showed p63 positivity, with relative sparing of syncytiotrophoblasts. Yolk sac tumors, embryonal carcinomas, and seminomas were negative for p63. Teratomas had occasional focal reactivity (32%) and strong staining intensity (mean, 2.9). All ETTs were positive for p63 (2+, 33%; 1+, 67%) with strong intensity of staining (mean, 2.7). p63 expression in the 2 cases of seminoma containing syncytiotrophoblasts was variable (3+, 50%; 0, 50%; mean intensity, 1.5).

3.4. β-Human chorionic gonadotropin

β-hCG demonstrated staining that frequently bled into surrounding tissue, necrosis, and hemorrhage, and often obscured cellular details, thus rendering it difficult to differentiate the tumor subtypes on the immunostained slide. Although reactivity was seen in almost every choriocarcinoma (96%), staining was present in all other tumor subtypes (embryonal carcinoma, 55%; yolk sac tumor, 47%; seminoma, 46%; teratoma, 32%; Tables 1 and 5, Figs. 1 and 2). Only one-third of ETT (2+, 33%) was positive for β-hCG. The syncytiotrophoblasts present in 2 cases of seminoma were positive for β-hCG (3+, 100%). For cases in which choriocarcinoma or

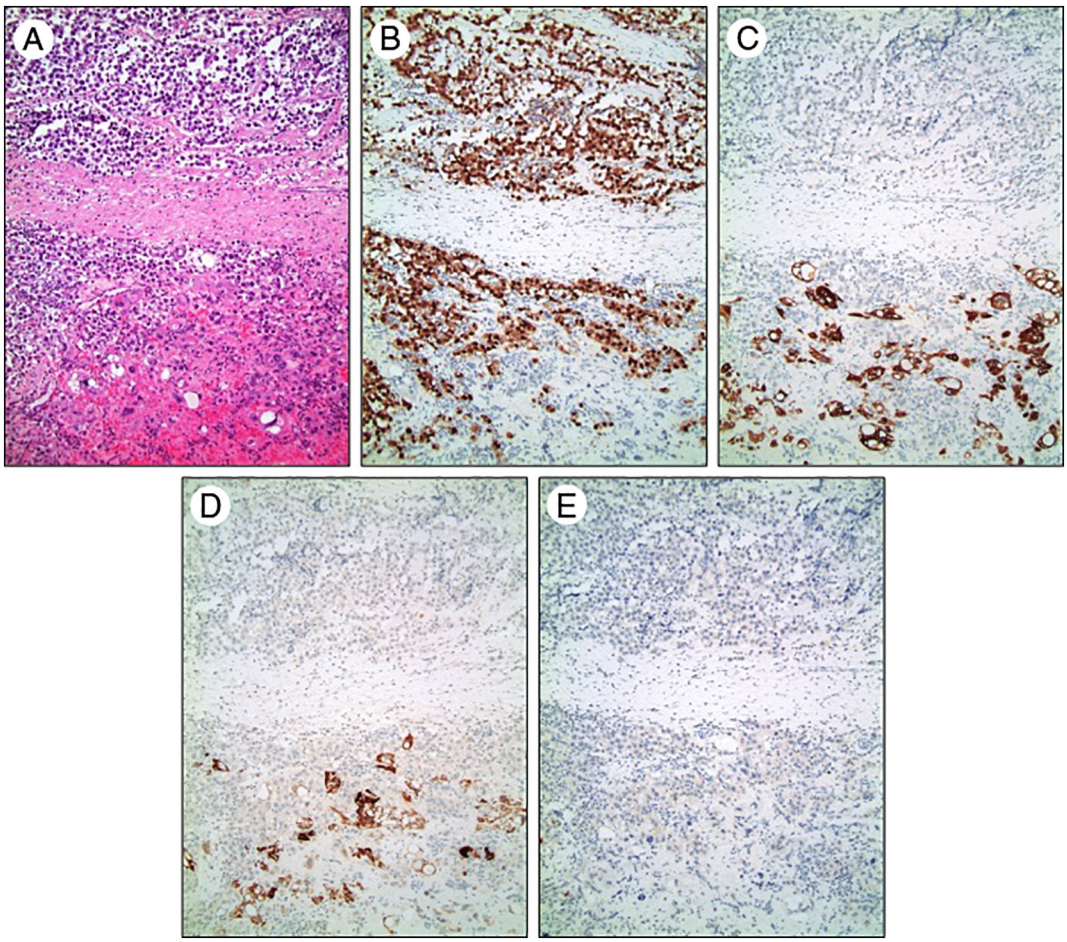

Fig. 3 Choriocarcinoma and seminoma in a testicle (original magnifications ×10 for all images). A, Hematoxylin and eosin staining with seminoma (top) and mixed seminoma and choriocarcinoma (bottom). B, OCT3/4, shown as a reference, with positive staining in seminoma and negative staining in choriocarcinoma. C, CK7 was positive in choriocarcinoma and negative in seminoma. D, Inhibin was positive in syncytiotrophoblasts of choriocarcinoma and negative in seminoma. E, p63 was negative in choriocarcinoma (with absence of mononucleated trophoblasts) and seminoma.

syncytiotrophoblasts were present in the tumor (either in the immunostained block or in another block), β-hCG was more likely to be positive in the other tumor subtypes ($P = .0002$). For embryonal carcinoma, 14 of 18 cases occurring in conjunction with choriocarcinoma or syncytiotrophoblastic cells were positive for β-hCG, whereas only 2 of 11 cases without choriocarcinoma or syncytiotrophoblasts were positive for β-hCG. Similarly, 7 of 10 yolk sac tumors, 6 of 13 teratomas, and 3 of 3 seminomas with choriocarcinoma or syncytiotrophoblasts present in the tumor were β-hCG positive, whereas 2 of 9 yolk sac tumors, 1 of 8 teratomas, and 11 of 25 seminomas without choriocarcinoma or syncytiotrophoblasts were β-

Table 3 Inhibin immunoreactivity in testicular GCT subtypes

Score (% of cells stained)	n (%)					
	CC	EC	YST	TR	SE	ETT
3+ (>50)	10 (37)	0	0	0	0	1 (33)
2+ (11-50)	11 (41)	0	0	0	0	1 (33)
1+ (5-10)	3 (11)	0	0	0	0	1 (33)
0 (<5)	3 (11)	29 (100)	19 (100)	22 (100)	28 (100)	0
Total no.	27	29	19	22	28	3
Mean intensity	2.7	–	1.0	1.0	–	2.7

Abbreviations: CC, choriocarcinoma; EC, embryonal carcinoma; ETT, epithelial trophoblastic tumor; SE, seminoma; TR, teratoma; YST, yolk sac tumor.

Table 4 p63 immunoreactivity in testicular GCT subtypes

Score (% of cells stained)	n (%)					
	CC	EC	YST	TR	SE	ETT
3+ (>50)	4 (15)	0	0	0	0	0
2+ (11-50)	13 (50)	0	0	0	0	1 (33)
1+ (5-10)	5 (19)	0	0	7 (32)	0	2 (67)
0 (<5)	4 (15)	29 (100)	19 (100)	15 (68)	28 (100)	0
Total no.	26	29	19	22	28	3
Mean intensity	2.7	–	2.8	2.9	–	2.7

Abbreviations: CC, choriocarcinoma; EC, embryonal carcinoma; ETT, epithelial trophoblastic tumor; SE, seminoma; TR, teratoma; YST, yolk sac tumor.

Table 5 β-hCG immunoreactivity in testicular GCT subtypes

Score (% of cells stained)	n (%)					
	CC	EC	YST	TR	SE	ETT
3+ (>50)	25 (93)	11 (36)	8 (42)	6 (27)	2 (7)	0
2+ (11-50)	1 (4)	3 (11)	0	1 (5)	4 (14)	1 (33)
1+ (5-10)	0	2 (7)	1 (5)	0	7 (26)	0
0 (<5)	1 (4)	13 (45)	10 (53)	15 (68)	15 (54)	2 (67)
Total no.	27	29	19	22	28	3
Mean intensity	2.6	2.3	2.6	2.1	1.6	1.0

Abbreviations: CC, choriocarcinoma; EC, embryonal carcinoma; ETT, epithelial trophoblastic tumor; SE, seminoma; TR, teratoma; YST, yolk sac tumor.

hCG positive. However, serum β-hCG was undetectable in 7 of 10 cases in which immunohistochemical β-hCG was positive in at least 1 tumor subtype without the presence of choriocarcinoma or syncytiotrophoblasts.

4. Discussion

The accurate identification and diagnosis of choriocarcinoma is imperative because it can affect the prognosis and treatment of testicular cancer [4]. Because of histologic overlap, differentiating choriocarcinoma from other subtypes of GCT can be difficult [2,3]. The metastatic setting can be particularly challenging because testicular GCT can metastasize with late recurrences distant from the testicle. In such cases, accurate diagnosis is vital to determine treatment. Currently, an ideal immunohistochemical marker for choriocarcinoma has not been elucidated. β-hCG is the most widely studied marker for choriocarcinoma but lacks specificity [2]. In addition, SALL4 has been shown to be helpful in identifying GCT in the metastatic setting but displays only variable positivity for choriocarcinoma and lacks specificity, as all other subtypes are reactive [5,6]. The markers CK7, inhibin, and p63 are widely available but poorly studied within testicular choriocarcinoma. Using both primary tumors and distant metastases, we aimed to determine if these markers were superior to β-hCG for the identification of testicular choriocarcinoma.

It is widely reported that β-hCG is expressed in testicular choriocarcinoma [7-12]. Consistent with the literature, we observed diffuse and strong expression of β-hCG in almost all choriocarcinoma, but reactivity often included abundant background staining diffusely throughout the tissue. All other subtypes, including seminoma, embryonal carcinoma, yolk sac tumor, teratoma, and ETT were also often positive for β-hCG, with a higher likelihood if choriocarcinoma was present in the tumor, consistent with the notion that secretion and subsequent diffusion of β-hCG cause this artifact. The high background and bleeding into surrounding tissue, necrosis, and serum made it difficult to identify, localize, and thus quantify tumor subtypes. Similarly, an investigation by Pelkey et al [13] using the same antibody utilized in our study also described nonspecific staining by β-hCG in placental, mediastinal, and testicular choriocarcinoma in dilutions up to 1:25 000, although only 1 testicular case was assessed. In addition to the more frequently seen diffusion artifact, we also noticed a subtler, less intense, patchy reactivity occurring occasionally in nonchoriocarcinoma subtypes in the absence of known β-hCG secreting cells (ie, choriocarcinoma or syncytiotrophoblasts), which seems to be nonspecific as serum β-hCG was undetectable in most of these cases.

Prior research has revealed immunohistochemical CK7 expression in placental tissue and choriocarcinoma cells lines [14,15], but no studies have evaluated the expression of CK7 in choriocarcinoma. We for the first time demonstrate that CK7 is strongly and diffusely positive in all cases of choriocarcinoma and ETT. In mixed GCT, CK7 staining allowed the choriocarcinoma component to be easily identified. It is important to be aware of the expression in choriocarcinoma as late metastases can be mistaken for a CK7-positive high-grade

carcinoma. Because seminomas were uniformly negative for CK7 and embryonal carcinomas exhibited only weak staining, CK7 effectively differentiated choriocarcinoma from seminoma and embryonal carcinoma. Similar to choriocarcinoma, yolk sac tumor expression of CK7 was often intense. Therefore, CK7 does not distinguish yolk sac tumor from choriocarcinoma. As expected, teratoma displayed variable CK7 positivity in the epithelial components of the tumor. In agreement with our results, Damjanov et al [14] reported seminoma to be completely negative for CK7, but Cheville et al [16] noted CK7 positivity in 41% of seminomas, although the expression was almost exclusively focal. Cheville et al [16] also identified CK7 reactivity in embryonal carcinoma, but the intensity of staining was not documented. Although CK7 has not been investigated in testicular yolk sac tumor, previous reports of ovarian yolk sac tumor have described negative or focal CK7 staining in 10% to 25% of cases [17-19].

We demonstrated that most cases of choriocarcinoma and all ETT strongly expressed inhibin with a variable percentage of cells positive. Inhibin expression in choriocarcinoma was generally restricted to the syncytiotrophoblasts, whereas mononucleated trophoblasts were usually negative. Because seminomas, embryonal carcinomas, yolk sac tumors, and teratomas were negative for inhibin, it is useful in differentiating choriocarcinoma from these subtypes. Inhibin is known to be produced by placental syncytiotrophoblasts, but only a few cases of testicular choriocarcinoma have been previously tested with similar results noted (n = 3, 100%; n = 1, 100%) [20-22]. In several prior analyses, inhibin was negative in testicular seminoma, embryonal carcinoma, yolk sac tumor, and teratoma [21-26]. A single study found inhibin positivity in 3 cases of testicular ETT [27]. Importantly, other tumors that may arise within or from the testes can also express inhibin. For example, cystic trophoblastic tumors (a subtype of GCT that may arise after treatment with chemotherapy), placental site trophoblastic tumors, and sex cord–stromal tumors have also been shown to be positive for inhibin [21,25-31].

In our study, most cases of choriocarcinoma and all cases of ETT were strongly positive for p63. In contrast to inhibin, p63 staining was predominantly observed in the mononucleated trophoblasts, with relative sparing of syncytiotrophoblasts within choriocarcinoma. Seminomas and embryonal carcinomas did not express p63. Most teratomas and a few yolk sac tumors exhibited focal and strong p63 reactivity, although all cases of yolk sac tumor were classified as negative owing to the positivity threshold that was used (≥5% of cells stained). Based on these results, p63 effectively differentiates choriocarcinoma from seminoma, embryonal carcinoma, and yolk sac tumor, but not teratoma. Normal placental cytotrophoblasts are known to produce p63, but this marker has been poorly studied in testicular GCT [32]. Two reports with a few cases have also described p63 expression in testicular choriocarcinoma to be generally localized to mononucleated trophoblasts (n = 1, 100%; n = 8, 100%) [33,34]. Similar to our results, 2 investigations demonstrated p63 negativity in seminomas and positivity in most teratomas and 25% to 40% of yolk sac tumors, although the extent of expression was not quantified [33,35]. The literature contains conflicting results on p63 reactivity in embryonal carcinoma. One analysis reported focal p63 positivity in 4 of 10 cases of embryonal carcinoma, whereas another found p63 to be negative in embryonal carcinoma (n = 4) [33,35]. In agreement with our study, Idrees et al [27] observed p63 expression in 4 of 4 testicular ETTs. It is relevant to note that cystic trophoblastic tumors have also been shown to display focal p63 reactivity in 2 of 6 cases and therefore must be considered in the differential when p63 expression is noted [28]. In addition, multiple case reports have described p63 negativity in testicular placental site trophoblastic tumors [27,29,30].

In conclusion, we demonstrate for the first time that CK7 is a highly sensitive marker for testicular choriocarcinoma and differentiates choriocarcinoma from seminoma and embryonal carcinoma, but not yolk sac tumor. Inhibin and p63 are both sensitive and specific for choriocarcinoma compared with seminoma, embryonal carcinoma, and yolk sac tumor. Because of the secretory nature of β-hCG yielding frequent background staining and nonspecific reactivity for other GCT subtypes, we recommend using CK7 with the addition of inhibin and p63 if markers are needed to differentiate and diagnose testicular choriocarcinoma. In the metastatic setting, it is important to note that choriocarcinoma has expression overlap with carcinomas of non-GCT origin, as this can confound the diagnosis in a distant, late metastasis.

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