# *Clinically atypical seminomas with yolk sac tumor features*

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***Introduction:*** *A small subset of young men die from seminoma. Studying these high risk, clinically atypical seminomas (CASs)—aggressive tumors with visceral metastases and chemotherapy resistance—may provide clues to the nature of drug resistance and the origin of testicular cancers. We explored the possibility that these seminomas are a unique clinical and biologic entity with intrinsic yolk sac tumor (YST) features.*

***Materials and methods:*** *We assayed available archived tissue samples (n = 22) for chemotherapy-resistance markers found in YSTs. Specifically, we analyzed tissues and clinical histories from patients with CASs (those who had visceral metastases and recurrent disease), classical seminomas, and mixed germ-cell tumors containing YST. By using immunohistochemical testing, we evaluated the expression of bone morphogenetic protein 2, alpha fetoprotein, and glutathione S-transferase (pi) [GST (pi)].*

***Results:*** *GST (pi) expression significantly predicted for overall survival (p = .036). In addition, according to the results of GST (pi) immunohistochemical staining, the CASs appeared to resemble YSTs more than they did classical seminomas (p = 0.043). Less-advanced tumors, both those that expressed GST (pi) and those that were negative for GST (pi), were more amenable to local therapies, and the patients who had those tumors had better clinical outcomes.*

***Conclusions:*** *Results from this exploratory study suggest that certain CASs that express GST (pi) are more similar to YST than they are to classical seminomas, and that GST (pi) expression may be able to be used as a prognosticator of disease-specific survival. Such CASs thus may have a unique biologic origin that differs from that of classical seminomas. Additional studies are needed to determine the natural history and therapeutic implications of these CASs.*

**Key Words:** clinically atypical seminoma, yolk sac tumor, testicular cancer, germ-cell tumor

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## Introduction

Classical seminomas are predictable: they metastasize to lymph nodes and are sensitive to radiation therapy and chemotherapy. However, certain seminomas have a propensity to metastasize to unusual sites in the visceral organs (e.g., brain or liver) and recur after cisplatin-based chemotherapy.[1–6] These clinically atypical seminomas (CASs) are potentially lethal. As such, they may teach us a great deal about the nature of drug resistance and the origin of testicular cancers.

Previous papers defined "atypical" seminomas as those that displayed the distinct metastatic features, chemotherapy resistance, and staining patterns of seminomas but had histologic characteristics of yolk sac tumors (YSTs).[7] The investigators of another prior study hypothesized that some seminomas

may be differentiated versions of seminomas with YST features.[8] Our previous work defined CASs as having high concentrations of β human chorionic gonadotropin (bHCG), visceral metastases, and/or chemotherapy resistance.[9] The results from that work demonstrated that bHCG was not predictive of, and pilot immunohistochemical analyses suggested that CASs were not related to, more undifferentiated tumor types. Therefore, we hypothesized that certain CASs, herein defined as having visceral metastases and/or chemotherapy resistance, are closely related to YSTs, and we speculated that validating this hypothesis will facilitate our finding a biomarker to distinguish these aggressive seminomas from the more easily treated "classical" seminomas.

In designing this exploratory experiment, we first conducted a literature search to identify markers that have previously been linked to YST and its various characteristics. Alpha fetoprotein (AFP) is a standard marker for differentiating YSTs from other germ-cell tumors. However, its expression would actually indicate a misdiagnosis in the case of an otherwise classical seminoma. Further, Looijenga et al[10] described the expression of bone morphogenetic protein 2 (BMP-2) in YSTs, which would explain the tendency of some YSTs to metastasize to bone. Because some CASs do migrate to bone, BMP-2 seems to be a good candidate as a marker of an association between these seminomas and YSTs.[5]

Another interesting marker, glutathione S-transferase (pi) [GST (pi)], has been linked to chemotherapy resistance in ovarian cancers and found to be uniformly involved in chemotherapy-resistant YSTs.[11,12] Considering these links to both chemotherapy resistance and YSTs, we hypothesized that GST (pi) is a useful marker for distinguishing between the aggressive CASs and the more treatable classical seminomas.

With this background in mind, we designed an experimental plan to test AFP, BMP-2, and GST (pi) by immunohistochemical analysis of any available tissues from patients with a seminoma of interest; as a comparison group, we used YSTs. Thus, we report here the results of immunohistochemical analysis for AFP, BMP-2, and GST (pi) on CASs, classical seminomas, and mixed germ-cell tumors containing YST.

## Materials and methods

### *Source of specimens and clinical data*

To identify available tissue samples for use in our analyses, we searched the data and information system in the Department of Genitourinary Medical Oncology at The University of Texas MD Anderson Cancer Center for three patient groups. We looked for patients with CASs with visceral metastases or chemotherapy resistance; a control group of classical seminomas, i.e. those that responded to treatment, had metastases only to lymph nodes, if any, and did not recur; and finally, a control group of mixed germ-cell tumors containing a YST component. Figure 1 provides details of the patients we identified.

The laboratory protocol for this study was approved by our institutional review board, and written informed consent to use their clinical data and archived specimens had previously been obtained from all patients.

### *Immunohistochemical testing*

Immunohistochemical analyses were done on 4-micron sections of each formalin-fixed paraffin-embedded archived tissue sample. Staining for AFP was done using anti-AFP antibody (1:3000; Zymed Laboratories, Carlsbad, CA, USA). In addition, the following primary antibodies were applied for standard DAB immunohistochemical analyses: BMP-2 (1:100; Abcam, Inc., Cambridge, MA, USA) and GST (pi) (1:25; Neomarkers, Inc., Fremont, CA, USA). Note that because of low amounts of antibody remaining for patient 3, GST (pi) staining was done manually by a histology technician who had no knowledge of the patient's case history.

After being stained with the antibodies, the slides were counterstained with Mayer's hematoxylin (Poly Scientific, Bayshore, NY, USA) and rinsed with water. The cells' nuclei were stained with bluing reagent (Richard-Allan Scientific, Inc., Kalamazoo, MI, USA), and then the specimens were dehydrated in consecutive baths of ethanol and xylene.

Positive-control specimens were stained in parallel with each set of antibodies. A standard clinically positive control was used for AFP, breast cancer tissue was used for BMP-2 and GST(pi),[13,14] and liver tissue was used as an additional positive control for GST (pi).

An Olympus BX41 microscope was used to view the slides at 200x magnification. The result of immunohistochemical staining was defined as positive if more than 25% of the tumor cells showed robustly positive cytoplasmic and/or nuclear signals, as focally positive if 5%-25% of the tumor cells showed such positive signals, and as negative if less than 5% of the tumor cells showed such positive signals.

### *Statistical methods*

We used Kaplan-Meier survival analysis and a log-rank test to compare overall survival and disease-specific survival between GST (pi)-positive and -negative groups. Fisher's exact testing was used to assess for an association between tumor type and GST(pi) expression

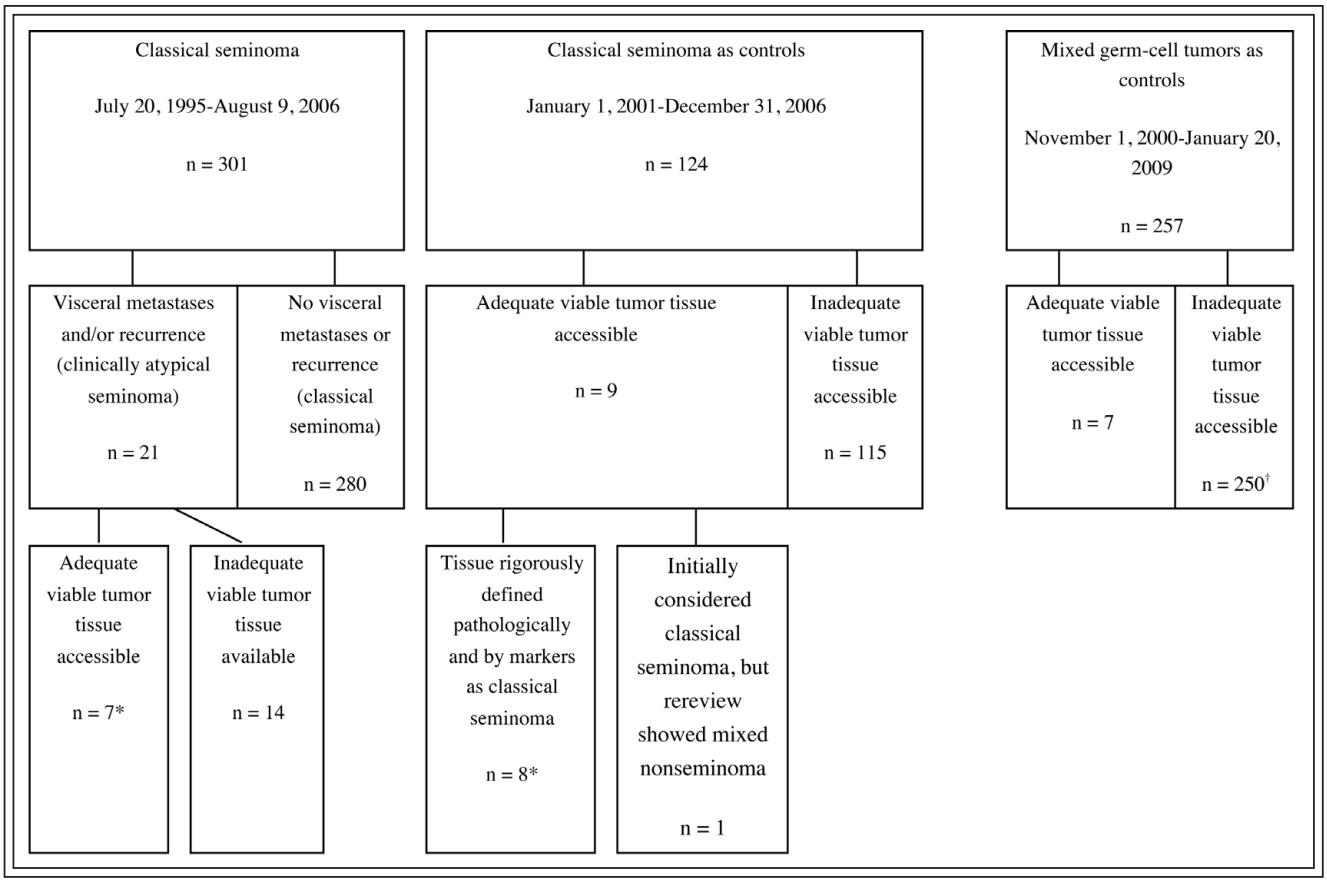


**Figure 1.** Description of specimens. *Only small numbers of archived tissues were available because some had been excised elsewhere. Our approved patient consent form applies only to tissues removed at our institution. †Most tissue samples were necrotic, thus unusable for staining.

with the help of our biostatistician and an online statistics calculator (http://in-silico.net/statistics/fisher_exact_test/2x3). Tumor types compared were CASs (those that metastasized to liver or bone), classical seminomas, and mixed germ-cell tumors with a component of YST. Statistical significance was set at $p \leq 0.05$.

## Results

### *Specimens*

Overall, we procured adequate tissue specimens from seven patients with seminoma plus visceral metastases (i.e., CASs), from eight with classical seminomas, and from seven with mixed germ-cell tumors containing some component of YST. Note that one patient identified as having classical seminoma was eventually found to have a microscopic focus of teratoma.

### *Immunohistochemical testing*

Positive staining for GST (pi) was found in the cytoplasm and nuclei of affected cells and also in normal Leydig and Sertoli cells within the vicinity of the tumor tissue, Figure 2. GST (pi) staining was positive in five of the seven patients with CAS, in three of the eight patients with classical seminoma, and in all seven patients with YST in mixed germ-cell tumors. Fisher's exact testing suggested that the CASs (i.e., those with metastases to the bone or liver) and YSTs were more likely to be positive for GST (pi) expression than were the classical seminomas ($p = 0.043$, Fisher's exact test 3 x 2). No significant difference in the expression of GST (pi) was detected between the CASs and the YST in mixed germ-cell tumors ($p < 0.46$, Fisher's exact test 2x2), however, suggesting that with respect to GST(pi), CASs stain more similarly to YSTs than they do to classical seminomas.

Positive BMP-2 staining occurred in the cytoplasm in one of the 15 patients with CASs or classical seminomas and in four of the seven patients with YST in mixed germ-cell tumors.

Positive AFP staining was found in none of the 15 patients with CAS or classical seminoma and in all seven of the seven patients with YST in mixed germ-cell

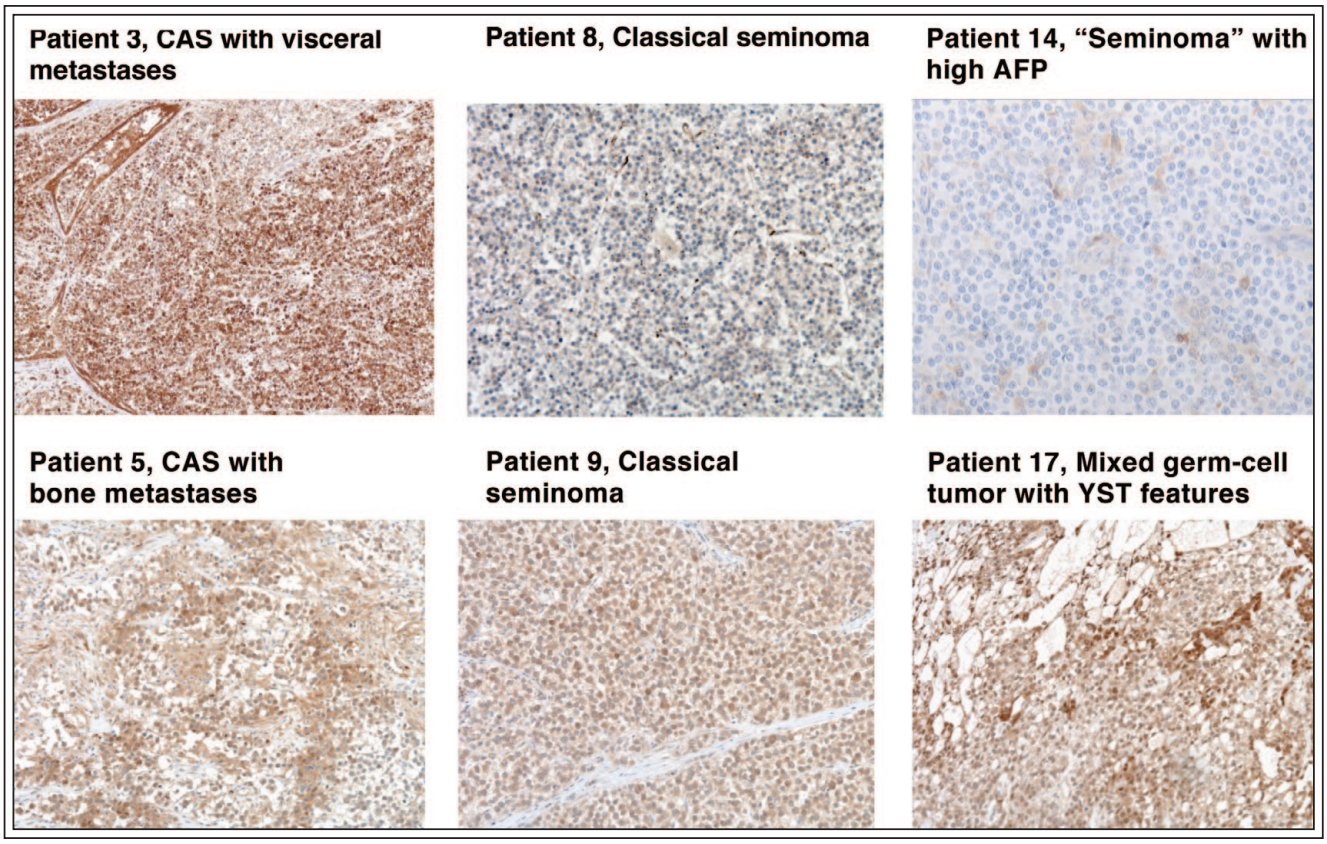


**Figure 2.** Representative GST (pi) staining. Patient 3, clinically atypical seminoma (CAS; visceral metastases). Patient 5, CAS (bone metastases). Patients 8, 9, and 14, classical seminoma. Patient 17, YST. Original magnification, 200×.

tumors. AFP positivity was found in both the cytoplasm and nuclei of the YST specimens.

### *Association between immunohistochemical staining and clinical characteristics*

We then determined whether there was an association between the different staining results and the patients' overall survival, treatment regimens, and location of the visceral metastases. Table 1 summarizes the clinical and staining data for our 15 cases of CASs and classical seminomas, and Table 2, the seven cases of mixed germ-cell tumors containing YST.

GST (pi) expression alone was a significant prognostic factor for seminomas (p = .036, Kaplan-Meier; Figure 3). Those patients with metastatic disease to the visceral organs died 14 to 23 months after the diagnosis. Of interest, the two other patients with CAS and metastases to the bone fared better. One responded to etoposide plus cisplatin and the other, to cyclophosphamide plus carboplatin. Post treatment, neither had any evidence of active disease. The tumor of one of those two patients stained positively for GST (pi). The presence of bone metastasis was not associated with BMP-2 expression in any of the tumors.

Of note, those patients with limited (or localized) as opposed to advanced seminoma have done well without regard to their GST (pi) status. All three patients with limited classical seminomas and tumors that stained positively for GST (pi) had undergone surgery, radiation, or chemotherapy. They did not succumb to their primary seminomas, although one patient who had undergone chemotherapy died of another unknown primary malignancy. It is important to note that all patients with tumors that did not express GST (pi) are currently disease free.

It is also interesting that the three patients with mixed germ-cell tumors that contained YST who died as a consequence of their disease also had advanced disease that was either not amenable to or did not benefit from surgery. As was the case with the patients whose advanced CAS stained positively for GST (pi), these three patients developed metastases in visceral organs and succumbed to their disease despite treatment, Table 2. In contrast, the patients with mixed germ-cell tumors that contained YST and stained positively for GST (pi) whose advanced disease was

TABLE 1. **Clinical data and markers detected in 15 cases of CASs and classical seminomas**

| Pt. No. | Tissue site | bHCG concentration (MIU/mL) | AFP concentration ng/mL | Visceral metastasis | Recurrence | BMP-2 staining | GST (pi) staining | AFP staining | Site of metastasis | Disease outcome | Survival time (mo) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | **Liver** | **3** | **2.8** | **Yes** | **Yes** | **Neg** | **Pos (30%), C, N** | **Neg** | **Liver, Nodal lungs** | **Not cured** | **19.3** |
| 2 | **RPLN** | **4.4** | **5.1** | **Yes** | **Yes** | **Pos (80%), C** | **Pos (90%), C, N** | **Neg** | **Liver Nodal** | **Not cured** | **19.2** |
| 3 | **Testis** | **147** | **5.2** | **Yes** | **Yes** | **Not done** | **Pos, C, N** | **Neg** | **Liver Nodal** | **Not cured** | **14.8** |
| 4 | **Testis** | **4.2** | **1.9** | **Yes** | **No** | **Neg** | **Neg** | **Neg** | **Bone, parasacral** | **Cured** | **169.5** |
| 5 | **Testis** | **18.8** | **2.6** | **Yes** | **No** | **Neg** | **Pos (30%), C, N** | **Neg** | **Bone** | **Cured** | **107.5** |
| 6 | **Testis** | **2.8** | **1.7** | **Not from seminoma** | **No** | **Neg** | **Neg** | **Neg** | **None** | **Cured** | **29.1** |
| 7 | **Testis** | **656.8** | **6.6** | **Yes (embryonal carcinoma)** | **Yes** | **Neg** | **Pos (30%), C, N** | **Neg** | **Nodal** | **Not cured** | **22.9** |
| 8 | Testis | 3179.7 | 6.2 | No | No | Neg | Neg | Neg | Nodal | Cured | 112.1 |
| 9 | RPLN | 9.3 | 6.6 | No | No | Neg | Pos (70%), C, N | Neg | Nodal | Cured | 42.5 |
| 10 | Testis | 57.2 | 2.9 | No | No | Neg | Pos (30%), C, N | Neg | Nodal | Cured | 67.8 |
| 11 | Testis | < 1 | 1.8 | No | No | Neg | Pos (80%), C, N | Neg | Nodal | Cured | 82.3 |
| 12 | Testis* | < 1 | 4.5 | No | No | Neg | Neg | Neg | None | Cured | 47.7 |
| 13 | Testis | < 1 | 5.4 | No | No | Neg | Neg | Neg | Nodal | Cured | 70.1 |
| 14 | Testis | 6.1 | 1.6 | No | No | Neg | Neg | Neg | None | Cured | 258.1 |
| 15 | Testis | < 1 | > 1000 | No | No | Neg | Neg (no tumor) | Neg | None | Cured | 272.2 |

Bold type indicates that seminoma was originally defined as "clinically atypical seminoma"; C = cytoplasmic; N = nuclear.; RPLN = retroperitoneal lymph nodes

*this patient was lost from follow up in 2 months. The Social Security Death Index was checked January 6, 2010, and he was not listed; we thus assumed that he was alive for at least the 6 months before we checked. †patient had a microscopic focus of teratoma.

TABLE 2. **Clinical data and markers detected in 6 cases of germ-cell tumors with YST features**

| Pt. No. | BMP-2 staining | GST (pi) staining | AFP staining | AFP concentration at diagnosis (ng/mL) | Pathologic composition of tumor | Patient status | Survival time (mo) |
|---|---|---|---|---|---|---|---|
| 16 | Pos/Neg (5%), C | Pos (50%), C | Pos (100%) | 859.6 | 100% YST | Deceased | 28.2 |
| 17 | Pos (30%), C | Pos (80%), C, N | Pos (80%) | 13455.7 | 80% YST, 19% embryonal carcinoma, 1% seminoma | Deceased | 75.8 |
| 18 | Pos (80%), C | Pos (100%), C, N | Pos (80%) | 1949.2 | 80% YST, 10% embryonal carcinoma, 5% seminoma, 5% mature teratoma | NED | 95.9 |
| 19 | Pos (20%), C | Pos (90%), C, N | Pos (40%) | 169.9 | YST and teratoma (majority teratoma, no % available) | NED | 104.3 |
| 20 | Pos (80%), C | Pos (100%), C, N | Pos (100%), C, N | 14131.9 | 100% YST | NED | 111.9 |
| 21 | Neg | Pos (90%), C, N | Pos (5%) | 103.9 | 10% YST, 60% embryonal carcinoma, 30% mature and immature teratoma | NED as of 3/25/2009 | 100.8 |
| 22 | Neg | Pos (90%), C, N | Pos (40%) | 6570.5 | 4% YST | Deceased | 21.8 |

C = cytoplasmic; N = nuclear; NED = no evidence of disease

amenable to surgery seemed to have benefited from therapy and experienced a better clinical outcome.

## Discussion

The results of this pilot study suggest that GST (pi) expression is useful for prognosticating disease-specific survival in a subset of seminomas, and as compared with certain advanced CASs, which by definition have a worse prognosis, are more similar to YST in their GST (pi) expression than classical seminomas are. We propose that these CASs belong to a novel subset of seminomas with unique biologic origins and properties.

GST (pi) is one of the xenobiotic-metabolizing and antioxidant enzymes. Its depletion has induced cell death, including apoptosis.[15] Overexpression of GST (pi) has been associated with carcinogenesis and the development of various human tumors and is often inversely associated with prognosis or patient survival. In particular, GST (pi) has been implicated in the acquisition of resistance to cisplatin by human testicular seminoma cells.[16]

YST is known to be a heterogeneous entity. Some YSTs resemble embryonal carcinoma and are easily eradicated with chemotherapy, whereas others behave more like teratomas and are relatively drug resistant. This clinical dichotomy of YSTs is also evident histologically: although classical YSTs display a well-described cystic pattern, others exhibit a solid cellular pattern. Indeed, the latter YSTs display the characteristics of a seminoma on light microscopy but have ultrastructural and functional characteristics somewhere on a continuum between seminoma and YST. According to Nazeer et al,[17] pure seminomas that have high concentrations of AFP might exist

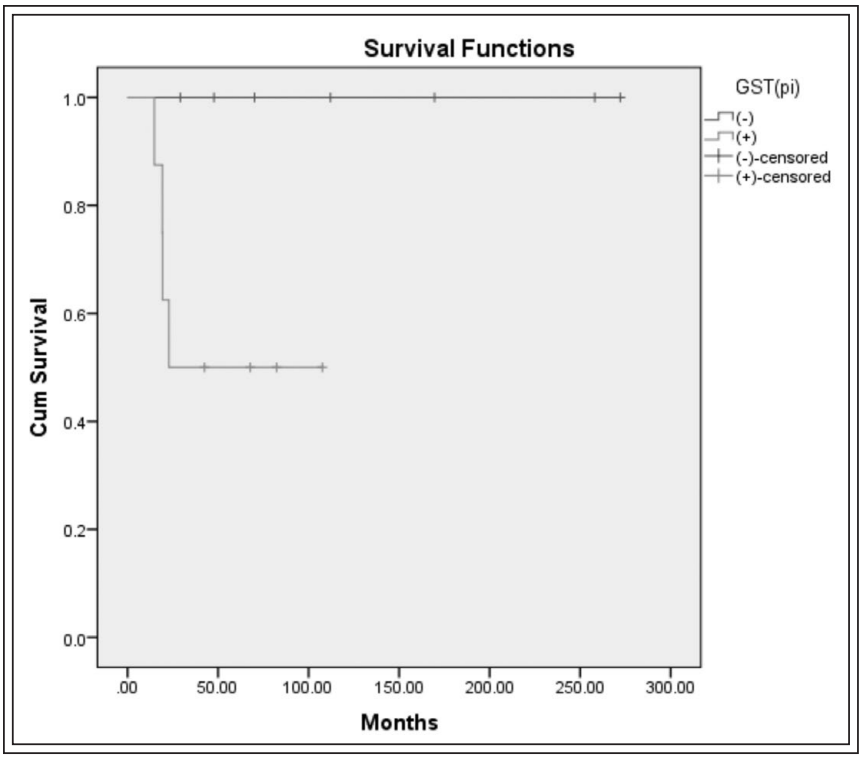


**Figure 3.** Disease-specific Kaplan-Meier survival curve by GST (pi) expression for 15 patients with seminoma. Data were current as of September 12, 2012.

but without any evidence of a hidden focus of YST. Furthermore, YST-like seminoma tumors may not necessarily express or produce AFP.[18]

Our analysis revealed evidence of a subgroup of seminomas that have features of CASs (i.e., with visceral metastases and chemotherapy resistance) and that resemble YSTs immunohistochemically by staining positively for GST (pi) but not for AFP. These findings suggest that CASs expressing GST (pi) represent distinct biologic or clinical entities that pursue distinct clinical courses and produce disparate clinical outcomes. We believe that the discovery of these novel biologic and clinical entities has important implications about the origin of germ-cell tumors in particular and of cancer in general.

Expression of GST (pi) during testicular carcinogenesis may be informative about the origin and nature of testicular cancer. For example, GSTs are important in normal spermatogenesis and in protecting germ cells from teratogens and carcinogens.[19] GST (pi) is strongly expressed in all elements of teratoma, irrespective of differentiation status.[12] The fact that GST (pi) is present in early progenitor/stem cells that have undergone some degree of differentiation but retain the drug-resistant phenotype has profound clinical implications.[18] It suggests that tumors derived from these early progenitor/stem cells possess intrinsic teratomatous or drug-resistant properties that render them difficult if not impossible to eradicate, especially at advanced disease stages.

One should emphasize that GST (pi) staining alone may not explain the chemotherapy-resistant properties of refractory seminomas. It is also plausible that GST (pi) does not play a causal role in the pathogenesis of CAS. This is evident by the positive staining for GST (pi) in both tumor and neighboring normal cells. Considering that most normal somatic cells and teratomas are also resistant to chemotherapy, we propose that expression of GST (pi) in those tumors is a marker of chemotherapy resistance, which can be overcome best by early surgical resection.

We believe that the existence of distinct subtypes of seminomas supports the stem-cell theory of cancers.[20] Many of us still envision the clonal evolution of germ-cell tumor from seminoma to nonseminoma,[21] whereas others envision a malignant precursor that develops into either a seminoma or a nonseminoma.[22] Our results, however, suggest an alternative model, in which discrete precursor cells in a stem-cell hierarchy give rise to either a mixed seminoma and nonseminoma or a pure seminoma.[20] Of note, Martineau[23] showed that in mixed tumors containing seminoma and teratoma, the two elements possessed similar karyotypes, implying a similar origin. On the other hand, in bilateral tumors of the testes, the tumors from separate testes were karyotypically distinct, suggesting a separate origin for the two tumor types.[23] Because the CASs originate from earlier gonadal stem cells (like their nonseminomatous germ-cell tumor counterparts), they tend to express a more heterogeneous phenotype than do classical seminomas, which are derived from later gonadal stem cells. Consequently, the CASs would have a distinct molecular signature [e.g., GST (pi) expression] that reflects their nonseminomatous characteristics (e.g., YST features) and that is different from those of the classical seminomas.

We emphasize that the results of this pilot study are intended to be only descriptive owing to the small

sample size. The sample size was limited as the result of the low incidence of CASs, lack of viable tumor after therapy, and restrictions from our institutional review board on obtaining tissues from outside institutions. The retrospective nature of this preliminary study also renders our results tentative because of potential bias or chance. Furthermore, no marker was known to differentiate seminomas before this exploratory study. Nevertheless, the idea that GST (pi) may be used to distinguish unique subtypes of seminomas (and perhaps other cancers) is novel and needs to be validated. We hope that future investigators may capitalize on our findings to launch a more robust study.

## Conclusion

In conclusion, the results of this exploratory study suggest that certain CASs that express GST (pi) are more similar to YSTs than they are to classical seminomas. Like YSTs, the CASs that express GST (pi) are more amenable to various therapies at an earlier stage. Such CASs thus may have a unique biologic origin that differs from that of classical seminomas. Additional studies are needed to determine the natural history and therapeutic implications of these CASs. □

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