Relation of Proliferative Activity to Survival in Patients with Advanced Germ Cell Cancer

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ABSTRACT

Patients with advanced disseminated germ cell tumors of the testis, retroperitoneum, and mediastinum have impaired survival compared to other patients with disseminated germ cell tumors having less bulky metastatic disease. Among patients with advanced disseminated germ cell tumors, we currently lack adequate predictors of long-term survival. Flow cytometric analysis of the paraffin-embedded, formalin-fixed tumor blocks of 50 of these patients suggests that proliferative activity is significantly correlated with survival \( (p < 0.001) \) in multivariate analysis. Log (beta-human chorionic gonadotropin) is the only other useful predictor of long-term survival in multivariate analysis of prognostic factors in this group of patients. Flow cytometric DNA analysis may be useful in predicting survival in patients with advanced disseminated germ cell tumors.

INTRODUCTION

The management of disseminated germ cell tumors has been one of the great successes of modern cancer therapy. With the introduction of cisplatin-based combination chemotherapy in the mid-1970s, more than half of all patients with disseminated GCT became potentially curable. Subsequent advances in management have reduced drug-related morbidity \( (e.g. \) with the substitution of vinblastine by etoposide) and improved complete remission rates \( (\) with surgical resection of residual disease, and with the addition of etoposide to cisplatin-containing regimens). Among patients with disseminated GCT, there are subgroups of patients with reduced survival. While these subgroups are defined differently in different staging systems, they have in common the presence of large volumes of metastatic cancer \( (1-4) \). In a recent review of patients with disseminated GCT treated at Indiana University, patients with either minimal or moderate volume of metastasis had long term survival rates of 98 and 90%, respectively \( (1) \). Among patients with advanced disseminated GCT \( (\) defined as patients with advanced pulmonary metastases, palpable abdominal mass plus pulmonary metastases, or hepatic, osseous, or central nervous system metastases \( ) \) only 58% of patients survived for more than 3 years \( \) (1). Patients with bulky disease therefore represent a continuing challenge for the oncologist.

The factors influencing survival within the group of patients with advanced disseminated GCT have been unclear. As all of these patients have relatively bulky disease, further subdivisions on the basis of clinical tumor mass have been un rewarding.

MATERIALS AND METHODS

Tumor Samples. Tumor samples utilized in this study were obtained from the archival formalin-fixed, paraffin-embedded specimens of the primary prechemotherapy lesions of patients with advanced disseminated GCT treated at Indiana University. All patients were entered on either Southeastern Cancer Study Group trial 78 GU 240 or on trial 82 GU 332. The former trial studied the role of maintenance chemotherapy in disseminated GCT \( (12) \). The latter trial randomized patients to receive either vinblastine or etoposide \( (VP-16) \) in combination with cisplatin and bleomycin, and demonstrated a survival benefit for patients with advanced disease receiving etoposide \( (13) \).

Processing of Specimens for Flow Cytometry. We employed the technique of Hedley et al., with minor modifications, in the processing of tumor samples \( (11) \). Multiple 50-\( \mu \)m thick sections were cut from paraffin blocks, deparaffinized with xylene, and rehydrated through graded alcohols. The samples were then digested with porcine pepsin (2500-3500 units/mg protein; Sigma Chemicals, St. Louis, MO) and then centrifuged over a 1 M sucrose gradient to remove debris. The nuclear pellet obtained was then resuspended in Tris-HCl buffer, pH 7.4, containing 50 \( \mu \)g/ml propidium iodide, 1 mg/ml ribonuclease A, and Nonidet P-40 (all obtained from Sigma). Following passage of the suspension through a 53-\( \mu \)m nylon mesh \( (\) Small Parts, Inc., Miami, FL), the nuclei were analyzed for DNA content in a Coulter 753 tunable dye laser flow cytometer with excitation at 488 nm. We attempted to perform flow cytometric analysis on as many tissue blocks as were available from each tumor.

Histopathology. Pathological analysis was performed on tumor sections immediately adjacent to the sections cut for flow cytometry. Routine 5-\( \mu \)m sections were cut and stained with hematoxylin and eosin for light microscopic examination. Flow cytometric analysis was performed preferentially on those blocks in which well-preserved neoplastic cells were the predominant population. Since the nonneoplastic elements consisted mainly of sparsely cellular connective tissues, the neoplastic cells usually contributed >85% of the nuclei in the sections. All specimens were classified histopathologically using the World Health Organization system of nomenclature.

Data Analysis. We quantitatively evaluated two flow cytometric parameters of DNA content: DNA index and PI. DNA index was defined as equaling the peak channel of the aneuploid Go/G1 peak divided by the peak channel of the euploid (2N) Go/G1 peak. Tumors containing a solitary Go/G1 peak were assigned a DNA index of 1.0.

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4. The abbreviations used are: GCT, germ cell tumor; PI, proliferative index; AFP, alpha-fetoprotein; hHCG, beta-human chorionic gonadotropin; LDH, lactate dehydrogenase.
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We did not attempt to evaluate DNA index in those apparently euploid (solitary Go/G1 peak) samples with excessively broad coefficients of variation of the G0/G1 peak, since we considered that such peaks might contain a near-euploid aneuploid peak beyond the limits of resolution of the flow cytometer. As previously described, proliferative indices were calculated in a manner similar to that of Naus et al. (14): by summing the counts in seven consecutive channels beginning with the third channel before the modal channel of the G1 peak and in seven consecutive channels beginning with the third channel before the modal channel of the G1 + M peak. The ratio of the latter to the former is expressed as a decimal fraction. In tumors containing euploid and aneuploid subpopulations, proliferative index analysis was performed on the aneuploid subpopulation. We have used this index in a previous publication (15). For purposes of statistical analysis within the series, when more than one PI index was obtained (from tumors with more than one tissue block), the PI used was the mean of the PI values for each block of that tumor.

Statistical analysis was performed using a generalized Wilcoxon test and a generalized Savage test for univariate analysis, and a stepwise Cox regression for multivariate analysis. The variables considered for multivariate analysis included tumor histopathology (utilizing a pathology score in which 1 = pure seminoma, 2 = mixed germ cell tumors containing seminoma, and 3 = nonseminomatous germ cell tumors), DNA index, PI (above or below the mean), number of elevated tumor markers (<3 versus 3), log (tumor marker), and treatment regimen (vinblastine-containing versus etoposide-containing). Student's t test was performed to compare the PI values of surviving and dying patients.

RESULTS

We performed flow cytometry on the neoplasms of 50 patients with advanced disseminated GCT. In all but two cases, analysis was performed on the primary testicular or mediastinal tumor; in the other two, only metastases to lymph nodes were available for study. A median of two blocks per case were processed (range, 1-4).

Histopathology. Pathological analysis was performed on all 50 specimens. The results of this analysis are shown in Table 1. The majority of our patients had mixed GCT (i.e., tumors containing more than one germ cell element). Comparing either pure seminomas to all other tumors, or pure seminomas plus mixed GCT containing seminomatous elements to all other tumors, we were unable to demonstrate any significant correlation between the pathological diagnosis and survival in univariate analysis.

DNA Index. We were able to compute DNA indices on 46 cases. The range of DNA indices is shown in Fig. 1. There was no significant correlation between DNA index and survival. The great majority of our patients had clearly defined aneuploid populations; only seven of our tumors contained solitary G0/G1 peaks. Three specimens contained more than two G0/G1 peaks (i.e., were multiploid) and three tumors were either tetraploid or near tetraploid, as defined by a prominent G0/G1 peak clustered near 4N, with an associated 8N peak. In this study, we found that the likelihood of discovering an aneuploid subpopulation increased with the number of tissue blocks analyzed, and that considerable heterogeneity of DNA indices was encountered when multiple blocks are processed for flow cytometry. An example of this heterogeneity is seen in Fig. 2.

Proliferative Index (PI). This study utilized PI rather than a determination of the percentage of cells in S-phase as an indicator of cell cycle kinetics. This was for two reasons. First, our experience with the Hedley technique has been that samples processed for flow cytometric analysis have higher percentage of S-phases than those encountered in fresh tumor specimens (data not shown). Second, on several occasions both G0/G1 peaks were associated with significant G2 + M peaks; the presence of a large euploid G2 + M population in the middle of an aneuploid S phase population made the S phase calculation difficult to perform. Haag et al., in a recent study of flow-cytometry-derived DNA histograms of breast cancer malignancies, have demonstrated a significant correlation between S-phase fraction and the G2 + M phase fractions (16). We were able to calculate a PI in 43 of the neoplasms. The data suggest an inverse relationship between PI and survival. Patients with PI values greater than the group had a mean survival of 37.7 ± 11.0 months compared with a mean survival of 85.6 ± 7.6 months for patients with PIs less than the group mean (p < 0.001 for both the generalized Wilcoxon test and the generalized Savage test). The relationship of PI to survival is shown graphically in Fig. 3.

Multivariate Analysis. Multivariate analysis of the effects of tumor pathology, DNA index, PI, treatment (cisplatin plus bleomycin plus vinblastine versus cisplatin plus bleomycin plus etoposide), and logarithms of the three tumor markers (AFP, βHCG, LDH) was done using a stepwise Cox regression to see if a combination of variables would predict survival better than a single variable. Results of multivariate analysis are shown in Table 2. Higher values for PI and log (HCG) are predictive of a worse outcome.

![Graph showing the relationship between DNA Index and survival.](image)

Fig. 1. Relationship of DNA Index to survival in patients with advanced disseminated germ cell tumors. DNA index (shown on the X axis) is calculated by dividing the aneuploid G0/G1 peak channel number by the euploid G0/G1 peak channel number. In tumors with no obvious aneuploid peak, DNA index equals 1.0. There is no significant relationship between DNA index and survival.

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Table 1: Histopathology of patients with advanced GCT

<table>
<thead>
<tr>
<th>Histologic type</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed GCT</td>
<td>29</td>
</tr>
<tr>
<td>Seminoma + embryonal carcinoma</td>
<td>6</td>
</tr>
<tr>
<td>Seminoma + embryonal carcinoma + immature teratoma</td>
<td>3</td>
</tr>
<tr>
<td>Seminoma + embryonal carcinoma + immature teratoma + yolk sac</td>
<td>2</td>
</tr>
<tr>
<td>Seminoma + embryonal carcinoma + yolk sac</td>
<td>1</td>
</tr>
<tr>
<td>Embryonal carcinoma + yolk sac</td>
<td>4</td>
</tr>
<tr>
<td>Embryonal carcinoma + immature teratoma</td>
<td>6</td>
</tr>
<tr>
<td>Embryonal carcinoma + immature teratoma + yolk sac + choriocarcinoma</td>
<td>4</td>
</tr>
<tr>
<td>Embryonal carcinoma + immature teratoma + non-GCT elements</td>
<td>1</td>
</tr>
<tr>
<td>Yolk sac + immature teratoma</td>
<td>2</td>
</tr>
<tr>
<td>Embryonal carcinoma</td>
<td>9</td>
</tr>
<tr>
<td>Seminoma</td>
<td>8</td>
</tr>
<tr>
<td>Teratoma (immune)</td>
<td>2</td>
</tr>
<tr>
<td>Choriocarcinoma</td>
<td>1</td>
</tr>
<tr>
<td>Yolk sac tumor</td>
<td>1</td>
</tr>
<tr>
<td>Unclassified</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
</tr>
</tbody>
</table>

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Table 2: Higher values for PI and log (HCG) are predictive of a worse outcome.
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Fig. 2. Morphological and flow cytometric heterogeneity in a patient with a mixed germ cell tumor. DNA histograms and paired tumor histopathology from three tissue blocks are shown. Top, a multiploid tumor (three G0/G1 peaks) from a block with embryonal carcinoma histopathology; middle, an apparently euploid DNA histogram from a tissue block showing seminoma; bottom, an aneuploid (but not multiploid) histogram from a block with embryonal carcinoma.

Fig. 3. Relation of PI to survival. Patients with a low PI have significantly ($p < 0.001$) better survival than patients with high PI. The figure represents a minimum follow-up time of 2 years and median follow-up of 41 months (range, 25-117 + months) following initiation of therapy.

Table 2 Multivariate analysis: Survival analysis for testicular cancer

<table>
<thead>
<tr>
<th>Variable</th>
<th>$p$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Log (HCG)</td>
<td>$0.016$</td>
</tr>
<tr>
<td>Pathology score</td>
<td>$0.045$</td>
</tr>
<tr>
<td>Treatment</td>
<td>NS*</td>
</tr>
<tr>
<td>DNA index</td>
<td>NS</td>
</tr>
</tbody>
</table>

* NS, not significant.

DISCUSSION

Previous flow cytometric studies of the DNA content of germ cell neoplasms have been few and have focused on ploidy without emphasis on the correlation of DNA content with survival. Zimmerman performed flow cytometric DNA histograms on 18 patients with malignant testis tumors (17). In his analysis, 17 patients had tumors containing aneuploid cell lines. The majority of patients in his study had earlier stages of disease than patients in this study, and he made no attempt to compare any index of proliferation with survival. Fossa et al. demonstrated aneuploidy in 19 of 20 primary testicular cancers analyzed, and found relatively high rates of proliferation (S-phase
between 22–51%) in seven of eight analyzable tumors (18). The small number of tumors with analyzable S-phases, and the fact that the majority of patients evaluated in this trial had early stage disease (hence good prognosis), precluded any analysis of the relation between any index of proliferation and survival (18). Barlogie demonstrated aneuploid subpopulations in 93% of 73 testis cancers discussed as part of a general review of flow cytometry-derived DNA content of human neoplasms (19). His review did not discuss the impact of cell proliferation on survival in GCT. Quirke et al. (20) analyzed the DNA indices of 61 patients with GCT, using both fresh and formalin-fixed (archival) tumor samples. Overall, 63% of seminomas, 68% of mixed embryonal carcinomas with teratomas, and 20% of tumors containing both seminoma and nonseminomatous elements contained aneuploid elements. The population of patients and neoplasms evaluated in this study is a large and relatively homogeneous group of patients with advanced stage disseminated germ cell tumors. All patients included in this study were treated at a single institution with similar cisplatin-based chemotherapy regimens. These factors allow for the investigation of the interrelationship of multiple biological parameters. The use of a technique allowing for the analysis of archival material allowed us to select a series of patients, all of whom had been followed for a minimum of 2 years. This minimum follow-up period is important in considering the results. In a recently completed review of 229 patients with disseminated GCT treated at Indiana University and followed for a median period of 102 months, 175 patients achieved complete remission. Of these patients, 27 have relapsed, with 20 relapses occurring in the first 24 months following completion of chemotherapy. Given the poor prognosis of all patients failing to achieve complete remission, a minimum follow-up of 24 months allows a correct determination of the ultimate prognosis of over 95% of patients with disseminated GCT.

Our data suggest a relation between tumor PI and survival. The patients with high proliferative indices (as indicated by a PI above the mean) have a significantly shorter mean survival than patients with whose tumors have low proliferative indices. Since our samples were (with two exceptions) obtained from the neoplastic primary, the data suggest that survival in advanced disseminated GCT may well be determined at a very early stage of the disease. A previously published analysis of southeastern Cancer Study Group patients with disseminated germ cell tumors suggested that the only predictor for survival among patients with advanced disease was the number of elevated serum markers (e.g., AFP, βHCG, LDH) (1). In our multivariate analysis, log (βHCG) predicted for survival, though less impressively (p = 0.016) than PI. Similar results have been seen in other studies of prognosis in germ cell cancer (21). Both factors [log (βHCG) and number of elevated markers] probably represent rough determinants of overall tumor mass.

It is interesting to compare our chemotherapy-era flow cytometry data with the prechemotherapy-era thymidine labeling data of Tubiana and Malaise, who found that embryonal carcinomas as a group had the highest thymidine uptake among five solid tumor histological types, and that tumor doubling time in these tumors was inversely correlated with patient survival (22). Our data suggest that the availability of curative chemotherapy has not altered the basic equation relating tumor growth rate and survival.

This analysis must necessarily be viewed with caution. Samples were not available for all Indiana University patients with advanced disseminated GCT entered on Southeastern Cancer Study Group trials 78 GU 240 and 82 GU 332. In some cases, the diagnosis of advanced disseminated GCT was made on the basis of either clinical picture alone (generally in patients presenting with life-threatening massive disease and elevated tumor markers) or by needle biopsy of a tumor mass. In other cases, specimen blocks were no longer available. The retrospective basis of this study naturally allowed us to perform flow cytometry only on the available tissue blocks, and this may represent a source of bias. Clearly, our results need to be confirmed in a prospective fashion which could eliminate or decrease potential sources of error.

We were unable to demonstrate any relation between DNA index and survival. This was not surprising. Our data, like that of Zimmerman and Foss et al. (17) suggest that the great preponderance of patients with GCT have tumors containing aneuploid subpopulations. Karyotypic analysis of germ cell tumors indeed has suggested that essentially all GCT are aneuploid regardless of tumor stage (23). GCT appear to be one of the few solid neoplasms in which aneuploidy is not correlated with impaired survival. Further, those tumors in our study with a DNA index of 1.0 are not necessarily euploid (2N) tumors. As mentioned above, we saw considerable heterogeneity between blocks, and there was a greater likelihood of detecting aneuploidy as more blocks were processed. For five of seven of our “euploid” specimens flow cytometric analyses were performed on only a single block; it is possible that with more blocks to process, we would have seen less euploidy. It is worth noting in this regard that Quirke et al. (20), analyzing 74 separate samples from 23 tumors, found heterogeneity of DNA content in seven of these 23. Furthermore, for reasons discussed by Hedley et al. (11), we lack a verifiable 2N standard in this technique. It is possible that the “euploid” solitary Ga/G, peaks seen in this study in fact represent homogeneous aneuploid tumor populations with undetectable levels of the 2N benign or normal tissue cells which usually allow one to distinguish euploid from aneuploid subpopulations.

In conclusion, our data suggest that GCT patients with elevated PI values have impaired long-term survival. This observation has potentially useful clinical implications. The volume of metastatic disease in patients with disseminated GCT clearly is prognostically important. However, advanced disease is quite heterogeneous both clinically and pathologically. The ability to identify patients with advanced disease which is likely to be responsive to first-line standard chemotherapy and distinguish them from those with advanced disease which is unlikely to respond, if confirmed in prospective studies, might allow the former to be spared unnecessarily toxic chemotherapy and afford the latter earlier initiation of more intensive therapy. We are developing such prospective studies to confirm our current retrospective observations.

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