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ORIGINAL ARTICLE

Androgen and c-Kit receptors in desmoplastic small round cell tumors resistant to chemotherapy: novel targets for therapy

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Abstract
Purpose Desmoplastic small round cell tumor (DSRCT) is a highly fatal, mainly peritoneal cell origin cancer which predominantly affects young adult males. This predilection in young males led us to examine the role of androgen receptors (AR), testosterone, and growth factors in the biology of DSRCT.
Methods Slides were prepared from 27 multi-institutional patients all with end-stage DSRCT. Slides were stained for AR, c-Kit, various growth factors, and drug resistance-associated proteins. Immunohistochemical (IHC) expression was scored semi-quantitatively. Western blot and MTT studies were performed to validate the IHC findings of over-expression of the AR and its functional status by stimulation of growth by dihydrotestosterone, respectively. Six patients with positive AR status were treated solely with combined androgen blockade (CAB) as used for prostate cancer.
Results Twenty-two patients were male (81%) and five were female (19%) with a median age at diagnosis of 23. All patients had failed at least two prior multi-agent chemotherapy regimens and 44% had progressed after autologous stem cell transplant. DSRCT samples from 10 of 27 patients were ≥2+ IHC positive for AR (37%, $P = 0.0045$) and 7 of 20 patients were ≥2+ IHC positive for c-Kit (35%, $P = 0.018$). We found elevated IHC expression of GST-pi, MRP and thymidylate synthase in smaller subsets of patients. In vitro studies for AR by Western blot and stimulation of growth by dihydrotestosterone in MTT assays suggest that the AR in DSRCT cells is functional. Six patients with positive AR status were treated with CAB alone and three

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of six attained clinical benefit (1-PR, 1-MR, 1-SD) in a range of 3–4 months. The three patients who responded to CAB had normal testosterone levels before CAB, while the three who did not respond to CAB had baseline castrate levels of testosterone.
Conclusions DSRCT has significant IHC expression of AR and c-Kit in heavily pre-treated patients. The presence of significant AR expression in 37% suggests that these patients could possibly respond to CAB. The significance of c-Kit expression in 35% of DSRCT patients is unknown and warrants further investigation.

Keywords Desmoplastic small round cell tumor · Androgen receptor · Testosterone · C-Kit

Introduction

Desmoplastic small round cell tumors (DSRCT) are rare and aggressive soft tissue malignancies that predominantly affect young adult males. The tumor was first described in 1991 [1]. It commonly presents with initial abdominal pain and distension. At diagnosis, the tumor is typically a large intra-abdominal or pelvic mass with regional dissemination or metastases to the liver, lungs, or bone [2]. Less commonly, DSRCT originates at extraperitoneal sites including the gonads and intracranial regions [3, 4]. The majority of DSRCT cases are distinguished by histology of solid clusters of undifferentiated small round cells embedded in dense desmoplastic stroma [5–7]. These tumors are also characterized by polyphenotypic differentiation as evidenced by immunohistochemical (IHC) staining for epithelial, mesenchymal, and neural markers including cytokeratins (EMA, AE1/3, CAM 5.2), desmin and vimentin, and neuron-specific enolase (NSE), respectively [8–10]. The tumor is also unique for a consistent reciprocal translocation t(11;22)(p13;q12) between the Ewing's sarcoma (EWS) gene on chromosome 22 and the Wilm's tumor (WT1) gene on chromosome 11, resulting in a EWS–WT1 fusion transcript [5, 10]. This fusion product causes a loss of the tumor suppressor function of WT1 and a putative upregulation of various families of growth factors from the EWS gene [5, 11, 12]. The recognition of this fusion product by RT-PCR is also a reliable diagnostic tool for DSRCT tumors arising outside of the abdomen or those with unusual morphological variations [10].

The prognosis and treatment of DSRCT remains a clinical challenge. Untreated DSRCT is commonly responsive to initial chemotherapy but frequently relapse with more resistant disease. Single and multi-agent chemotherapy trials have yielded moderate results, except for the highly active P6 regimen developed by Kuschner et al. [13] consisting of cyclophosphamide, doxorubicin, vincristine, ifosfamide, etoposide, and MESNA. Despite advances in chemotherapy and stem cell transplantation, DSRCT is commonly fatal with a median survival of 2.5 years [4, 14, 15].

Here, we report the results of a study that investigated the presence of various growth factors and drug resistance-associated proteins in 27 patients with DSRCT. We previously noted the association between DSRCT in young males to rising testosterone levels, which prompted the investigation into the role of androgens in the biology of DSRCT. Additionally, we report the anecdotal results of a pilot clinical study that utilized combined androgen blockade (CAB) in six DSRCT patients with recurrent, drug-resistant disease.

Patients and methods

Patients and tissue

The 27 DSRCT cases collected in this study from 1999 to 2005 were obtained from the consultation files of one of the authors (Robert L. Fine). Slides were prepared using a coded method in accordance with HIPAA regulations. Patient information was obtained from referring pathologists and oncologists. All living patients gave written consent for their tumors to be studied. Compassionate exemption was obtained for CAB treatment from respective IRBs if the patients were offered hormonal therapy (six patients at four hospitals). The tumor tissues tested were from the last surgery performed for therapeutic reasons. All patients were examined at the Columbia University Medical Center (by Robert L. Fine) or had their records examined at least once at Columbia.

Four-micrometer sections were prepared from paraffin-embedded tumor blocks from the last tissue collection. All tissues studied were from patients who had tumor progression after at least two multi-agent chemotherapy regimens (100%) and/or autologous stem cell transplant (44% ASCT). Sections were mechanically dissected and digested with DNase and collagenase. Disaggregated tumor cells in suspension were spun onto glass slides by cytocentrifugation. Samples were stained with H&E, Giemsa, and special stains including desmin, actin, NSE, and vimentin. All immunohistochemical (IHC) preparations and scoring were performed by an investigator (Ing-Ru Yu) and a board-certified Pathologist (Michael Richadson) at Oncotech, Inc., who were not blinded to the diagnosis of DSRCT. All samples were reviewed a second time

to confirm a tissue diagnosis of DSRCT at Oncotech Inc. by a board-certified Pathologist (Michael Richadson), and by the primary Pathologist. The diagnoses of two patient samples that were treated with CAB were confirmed with the identification of the EWS–WT1 fusion product by RT-PCR.

IHC panel

The IHC panel included antibodies to: androgen receptors (AR), c-Kit (CD 117) receptors (antibody recognizes wild-type and mutant c-Kit), various growth factor receptors for: estrogen (ER), progesterone (PR), Her-2-neu, epidermal growth factor (EGFr), and drug resistance-associated proteins: Bcl-2, pi isoform of glutathione-*S*-transferase (GST-pi), methyl guanylmethyl transferase (MGMT) (0^6-AGAT), multidrug resistance protein 1 (MDR1), MRP, Bax, thymidylate synthase (TS), and p53. All these IHC antibodies utilized in the study were monoclonal except the polyclonal antibody CD 117 to c-KIT receptor. Following cytocentrifugation, antigen retrievel was maximized with microwave heating and appropriate enzymatic digestion. IHC staining was then performed using Biogenex and Ventana autostainers at Oncotech Inc.

Positive immunoreactivity for the IHC studies was semi-quantitatively scored based on a score of greater than or equal to 2, which is the sum total of staining intensity (0–500) divided by percent positive cells (0–100%) minus 1.0. The sample staining score was as follows: 0 = no staining; 1+ = indeterminate value; 2+ = positive; 3+ = moderately positive; and or 4+ = highly positive. The highest score achievable in this semi-quantitative analysis was 4+ (highly positive). Only scores $\geq$2 were considered positive for presence of the antigen tested. At least four separate slides from the same tissue block were examined, scored and the mean score derived from these samples.

Laboratory in vitro studies

Androgen receptor

Standard Western blots were performed on tumor cell lysates from the malignant ascites of two patients at two separate points of time to corroborate the IHC presence of AR. Ascitic DSRCT cells were isolated and concentrated (>90%) by repeated Ficoll-Hypaque gradient separations. Tumor samples tested were: (1) prior to CAB therapy and (2) at relapse while on CAB. The Western blot method followed our previously published method [16] and utilized a different monoclonal AR antibody, N-20 (Santa Cruz Biotech. Santa Cruz, CA, USA) than the monoclonal antibody used in IHC studies (F39.4.1; BioGenex). Blots were performed twice for each sample.

Cell proliferation assays

Disaggregated DSRCT cells were obtained from the malignant ascites from one patient before CAB was initiated. Once grown in the media stated and pathologically confirmed as DSRCT, cells were plated in a standard MTT assay following the methodology we previously published [17]. After plating the cells for 18 hr in RPMI 1640 media supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine, dihydrotestosterone (DHT) was added $\pm$ flutamide, each at 50 nM. Four days later, the plates were harvested and cell viability and growth were assessed. All experiments were performed twice in triplicate.

Statistical analysis

All *P* values for AR and c-Kit IHC scores were calculated by the Wilcoxon Signed Rank Test and were one-tailed tests, given expected IHC data to be unidirectional in showing no staining for AR or c-Kit. In calculating these values, no previous AR expression data existed for DSRCT tissue and our null hypothesis assumed AR expression to be zero. c-Kit expression data was available from two previous IHC studies [10, 18]. These two published IHC studies in DSRCT, however, included mainly untreated, chemo-naive DSRCT patients (>80%) [10, 18] in contrast to this study, which utilized 100% of samples from relapsed, heavily pretreated patients.

Spearman's rank correlation coefficients were used to identify correlations between c-Kit and AR. Also, AR and c-Kit correlations were analyzed between these two variables individually and with the following variables: sex, age, ASCT treatment, $\geq$ 2+ receptor status for: estrogen, progesterone, Her-2-neu, EGF, and presence of: Bcl-2, GST-pi, MGMT (0^6-AGAT), MDR1, MRP, Bax, TS, and p53 expression.

Pilot clinical trial

Six living patients from four institutions with positive AR status (all 3–4+) were identified from other patients who were AR positive (total of ten patients). These six patients were chosen because they had no further standard therapeutic options. They had progressed despite surgery, radiation and extensive chemotherapy including two patients who progressed

after ASCT. Each had end-stage cancer and demonstrated progressive disease on recent CAT scans. The patients were treated by their primary oncologist or at Columbia. Patients were required to have a 3- and 4-week hiatus off of all chemotherapy and radiation, respectively. Levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), serum testosterone, and DHT were determined prior to initiation of CAB and at monthly intervals following initiation of CAB. Serum testosterone levels were considered castrate if they were below the lower limits of normal. CAB therapy consisted of an AR blocker, biculatamide 50 mg po qd for 1 week preceding Lupron (7.5 mg i.m. monthly), both administered for at least 1 month. CAB therapy was given as a singular treatment without any concurrent chemotherapy, radiation, surgery, hormonal, or holistic therapies. Disease progression, stability, or response was determined monthly after at least 1 month of CAB. Standard WHO criteria for response, stable disease, or progression were utilized for evaluation requirements. Therapy was continued depending on the duration of individual clinical responses.

Results

Clinical features

Twenty-two patients were males (81%) and five were females (19%). The median age at diagnosis was 23 with a range of 4 months to 74 years. The predominant site of tumor involvement at the time of diagnosis was the abdominal cavity (20/27 cases, 74%) with individual cases detected within the lungs (2), pleura (1), posterior ear (1), breast (1), subcutaneous tissue (1), and cervical spinal cord (1). All living patients (20/27) had end-stage DSRCT with an ECOG score of 3. Prior clinical treatments included failure of at least two chemotherapeutic regimens (100% of patients), including in most patients the P6 regimen and 44% had failed ASCT with high-dose myeloablative chemotherapy, consisting mainly of alkylators.

Immunohistochemistry

The IHC analyses of 27 DSRCT cases are shown in Table 1. Ten of 27 samples stained positive (≥2+) for AR (37%, $P = 0.0045$). Of these ten positive specimens of 27 total patient samples, one sample stained highly positive (4+, 3.7%); eight stained moderately positive (3+, 29.6%), and one stained positive (2+, 3.7%). Seven of 20 samples stained positive (≥ 2+) for c-Kit (CD 117) (35%, $P = 0.018$). Of these, four of the samples stained moderately positive (3+, 20%) and three stained positive (2+, 15%). Staining for growth factor receptors including ER, PR, Her-2/neu, and EGFr were largely negative with 2/12 cases staining positive (≥2+) for EGFr (17%) and with 3/14 cases staining positive (≥2+) for Her-2/Neu (21%). Though the following number of cases was small, staining for drug resistance-associated proteins in DSRCT yielded high expression (3+) of GST-pi (3/9), MRP (4/6), and TS (4/6). The others showed the following staining of ≥ 2+: MGMT (0^6-AGAT) (3/9), MDR1 (2/15), p53 (5/11), Bax (2/3), and Bcl-2 (3/7) (Table 1).

In correlation coefficient analysis, no correlation was detected between positive or negative AR and c-Kit status. There was no correlation between AR or c-Kit status and: age, sex, ASCT, ER, PR, EGFr, Her-2-neu, GST-pi, MRP, TS, Bcl-2, MGMT (0^6-AGAT), MDR1, p53, and Bax expression.

Table 1 Immunohistochemical (IHC) studies of 27 cases of desmoplastic small round cell tumors (DSRCT)

IHC	AR	CD117 (c-Kit)	ER	PR	HER-2-neu	EGFr	GST-pi	MRP	TS	MGMT (O^6-AGAT)	MDR1	p53	Bax	Bcl2
n =	27	20	12	12	14	12	9	6	6	9	15	11	3	7
0	15	13	12	12	10	10	3	2	1	3	11	5	1	4
1+	2	0	0	0	1	0	1	0	0	3	2	1	0	0
2+	1	3	0	0	2	1	2	0	1	1	1	3	0	1
3+	8	4	0	0	1	1	3	4	4	2	1	2	1	2
4+	1	0	0	0	0	0	0	0	0	0	0	0	1	0
	10/27	7/20	0/12	0/12	3/14	2/12	5/9	4/6	5/6	3/9	2/15	5/11	2/3	3/7
Total+	37%	35%	0%	0%	21%	17%	56%	67%	83%	33%	13%	45%	67%	43%

0, negative; 1+, indeterminate; 2+, positive; 3+, moderately positive; 4+ highly positive

AR androgen receptor; *TS* thymidylate synthase; *ER* estrogen receptor; *PR* progesterone receptor; *EGFr* epidermal growth factor receptor; *GST-pi* glutathione-*S*-transferase pi isoform; *MGMT* methyl guanylmethyl transferase (a.k.a. O^6-AGAT); *MDR-1* multidrug resistance type 1

In vitro studies

Androgen receptor

We obtained viable DSRCT cells twice from malignant ascites from case 2 (patient #12) and case 3 (patient #15) described next. We confirmed IHC positivity for AR (3+) in patient #12 by Western blot at two time points in his clinical course: (1) prior to CAB and (2) at relapse 4 months later on CAB. Figure 1 (lane 1) shows a prominent band at the molecular weight for the AR by the specific monoclonal antibody for AR, which corroborated the positive IHC staining results (3+). At relapse, the AR band was not detectable by Western Blot (Fig. 1, lane 2) or by IHC analysis. This suggested that the AR was downregulated or lost in ascitic DSRCT cells from the same patient in relapsed, growing tumor while on treatment with CAB 4 months later.

MTT assay

Primary in vitro cultures from patient #12 (case 2 above) before initiation of CAB were also established. Figure 2 shows the growth levels of Ficoll-Hypaque separated ascitic tumor cells from the initial sample (prior to CAB), which had demonstrated the presence of AR in Western blot (Fig. 1, lane 1) and by IHC (3+). These primary cultures were cytologically confirmed to be DSRCT (>90% of the sample) by a Pathologist at Columbia (Hanina Hibshoosh). All cells were grown in standard RPMI 1640 media with 10% heat inactivated FCS. Figure 2 shows the results of the following experimental groups: Group 1 = untreated; Group 2 = supplemented with 50 nM of DHT alone; Group 3 = supplemented with 50 nM of the AR blocker flutamide alone; and Group 4 = supplemented with DHT and flutamide, both at 50 nM. By day 4 of the MTT assay, DHT alone stimulated cell growth by 300% above the control RPMI media with 10% FCS. Flutamide alone reduced the growth of the DSRCT cells by 62% below the control group (RPMI media with 10% FCS). The addition of flutamide with DHT reduced the stimulation of growth by DHT from 300 to 42% above control values. These studies were performed two times each in triplicate with mean and standard error of the mean (SEM) shown in Fig. 2.

Outcome of pilot clinical study

Nine males (9/22) and a single female (1/5) patient had positive AR status (≥ 2+) in IHC. The definition of clinical tumor benefit includes: complete (CR)/partial (PR)/minor responses (MR) and stable disease (SD) by standard WHO criteria. CAB of six patients (five males, one female) with positive AR status yielded three male patients with a clinical tumor benefit (Table 2). Two patients showed a partial and minor response in lung and abdominal masses for a period lasting 3 and 4 months to CAB alone, respectively. The third patient had SD for 3 months on CAB alone of baseline progressive pleural based masses. All three

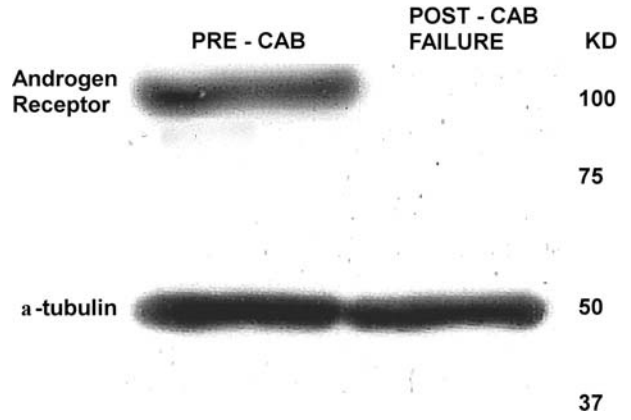

Fig. 1 Representative Western blot showing presence of androgen receptor (*AR*) (100 kDa) in ascitic desmoplastic small round cell tumor (*DSRCT*) cells (50 ug of homogenate) from patient #12 prior to initiation of combined androgen blockade (*CAB*) therapy (*left lane*). Downregulation or loss of AR expression in ascitic DSRCT cells taken from the same patient at CAB failure is indicated by absence of AR band (*right lane*). Alpha tubulin staining was used for loading controls. Western blots were performed twice

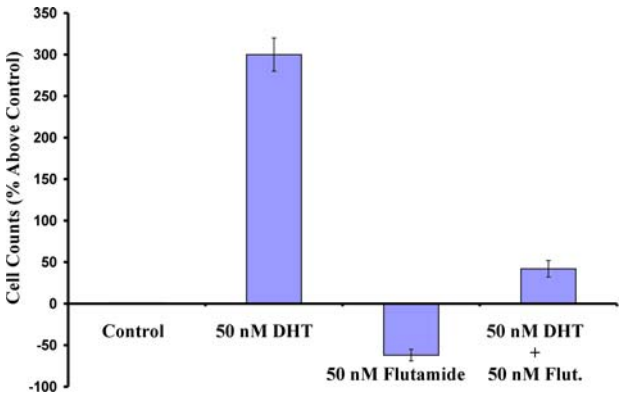

Fig. 2 In vitro growth of androgen receptor (AR)-positive desmoplastic small round cell tumor (*DSRCT*) cells in MTT assays from patient #12 at 4 days after exposure to 50 nM of either dihydrotestosterone (*DHT*) alone, flutamide alone, and both DHT and flutamide. These DSRCT cells were obtained from malignant ascites before combined androgen blockade (*CAB*) and are from the tumor cells in Fig. 1, which show a positive AR band (*left lane*). The *graph* demonstrates inhibition of basal and DHT-stimulated DSRCT cells by the androgen receptor blocker flutamide. The *bars* and standard error of mean (*SEM*) were derived from two experiments performed in triplicate

Table 2 Cases treated with combined androgen blockade (CAB)

Case no.	Prior treatment regimens[a]	AR status and score	Baseline testosterone levels	CAB outcome
12	P6, CPT-11, Thalidomide, XRT, MTX	Positive/3+	Normal	MR for 4 months
15	P6, Pacl/Carbo, VP-16, Epothilone/DOX	Positive/3+	Normal	PR for 3 months
21	P6, ASCT with Thiotepa/Carbo, XRT, CPT-11	Positive/3+	Normal	SD for 3 months
1	P6, VP-16, Pacl/CDDP	Positive/3+	Castrate	NR
4	Carbo, P6, CTX/Topotecan, ASCT, XRT	Positive/3+	Castrate	NR
14	VP-16, CDDP, CTX, VCR, XRT	Positive/4+	Castrate	NR

[a] Chemotherapy agents given together are marked by a slant (/). *ASCT* autologous stem cell transplant; *PR* partial response; *MR* minor response; *SD* stable disease; *NR* no response; *XRT* radiation therapy; *CTX* cyclophosphamide; *MTX* methotrexate; *DOX* doxorubicin; *VCR* vincristine; *Carbo* carboplatin; *Pacl* paclitaxel; *CDDP* cisplatin; *VP-16* = etoposide

patients who had clinical tumor benefit had normal testosterone levels at the initiation of CAB therapy. All three CAB failures (two males and one female) had castrate levels of testosterone at the start of CAB alone therapy (Table 2). A brief synopsis of the responders is given next.

Case 1 Patient 21 is a 35-year-old male diagnosed with DSRCT in February 1998. Patient's treatment prior to CAB included three separate treatments: (1) the P6 protocol followed by surgical debulking; (2) ASCT (high-dose thiotepa and carboplatin) with radiation therapy; and (3) CPT-11. He had partial remissions to each treatment. In December 2001, he demonstrated progressive pleural masses on CPT-11 therapy. The pleural masses showed 3+ AR expression and 3+ HER-2-neu expression with normal baseline testosterone levels. He was initiated on CAB for 4 months in early 2002 and showed stable disease for a 3-month duration

Case 2 Patient 12 was a 17-year-old healthy male who presented with metastatic DSRCT in June 1999. Molecular diagnosis was made with the identification of the chimeric EWS-WT1 fusion by RT-PCR. The patient underwent seven cycles of the P6 protocol with aggressive thoracic/abdominal surgical debulking, but continued to have residual disease. He received various regimens including: CPT-11; thalidomide; holistic medicines with radiation; and experimental vaccines, each producing no significant tumor regression. In June 2000, he presented with multiple brain and vertebral-spinal metastases. Patient received whole brain XRT and in 2000 he was transferred to Columbia University Medical Center and received intrathecal methotrexate and later Ara-C via an Ommaya reservoir. His neurologic symptoms improved but developed metastases to bone, kidney, lungs, peritoneum and development of malignant ascites. His course was then complicated by fever, sepsis and respiratory failure requiring transient intubation, antibiotics and pressors. Bronchoscopy revealed extensive metastases of tumor to the intra-bronchial tree and bronchial biopsy revealed DSRCT. The ascitic tumor cells revealed 3+ positive AR on IHC and a prominent band for AR on Western blot in our lab (Fig. 1, lane 1). He concomitantly had normal serum testosterone levels. The ascitic tumor cells in MTT assays demonstrated stimulation of growth by DHT and suppression of growth by flutamide (Fig. 2). He was started on CAB alone and repeat bronchoscopy 4 weeks later revealed marked regression of tumor lining the bronchi (>50%) and resolution of ascites. Solid tumor masses at other sites regressed less than 50% but more than 25% by WHO criteria. However, there were multiple perforations in the bronchial tree left by receding tumor, leading to multiple bouts of sepsis from a pulmonary origin. After 4 months of CAB, evidence for disease progression was noted with return of malignant ascites and a paracentesis was performed. Repeat Western blot on the ascitic tumor cells demonstrated loss of the AR while on CAB treatment (Fig. 1, lane 2). This minor response on CAB lasted 4 months; however, the patient succumbed to progressive disease and pulmonary sepsis.

Case 3 Patient 15 was a 21-year old healthy male who presented in January 2000 with liver metastases and ascites from DSRCT. Diagnosis was later confirmed with the identification of the chimeric EWS–WT1 fusion by RT-PCR. The patient was treated with the P6 Protocol for four cycles with a partial response but progressed. The patient then received paclitaxel, carboplatin and VP-16 without effect; followed by stable disease for 2 months from epothilone and doxorubicin. In September 2000, he developed malignant ascites from progressive tumor and had 3+ AR IHC expression of his ascitic tumor with concomitant normal testosterone levels. He was treated with CAB alone for 4 months.

The patient experienced a major reduction in pain symptoms and analgesic use lasting 3 months. Follow-up positron emission tomography (PET) imaging showed a 75% decrease in PET avidity of liver metastases and a contrast CT scan showed greater than 50% reduction of all hepatic metastases for 3 months following CAB. In early 2001, patient developed recurrent ascites, which by IHC revealed loss of AR expression (0+) with castrate testosterone levels. Recurrent tumor also revealed new c-Kit expression (3+) by IHC that was negative prior to CAB. The patient was treated with Imatinib mesylate without a response and succumbed to progressive metastatic disease in June 2001.

Discussion

Patients with DSRCT are usually sensitive to chemotherapy at presentation; however, they commonly have disease recurrence, widespread metastases, and in the great majority of cases, succumb to the cancer despite intensive chemotherapy and ASCT [9, 19, 20]. Aggressive surgical resection of gross tumor is one of the major mainstays of treatment that improves progression-free survival [2, 13, 19]. Consolidative radiation therapy effectively improves the local control of DSRCT [9, 15, 21] while multi-agent chemotherapy regimens for DSRCT at high doses (P6 protocol) have generally induced substantial initial remissions [23]. Aggressive combined modality treatments have also offered improvements in patient survival. In one series by Schwarz et al. [19], aggressive surgical debulking, radiation therapy, and the P6 protocol resulted in an overall progression-free survival of 18% at 5 years. In an earlier study by Kushner et al. [13], 12 patients receieved the P6 protocol, followed by tumor resection or radiotherapy and/or ASCT with high-dose thiotepa and carboplatin resulting in seven complete clinical remissions (7/12, 58%). Other chemotherapy trials [2, 4, 23] have offered little survival advantage over the P6 protocol, while the true benefit of myeloablative ASCT therapy on survival remains unanswered. In this context, there is a need for the identification of new DSRCT targets and the development of directed therapeutic regimens especially for relapsed, heavily pre-treated patients.

Androgen and c-Kit receptor status

DSRCT has a 90% predilection for young adult males who normally have rising or high testosterone levels, which led us to hypothesize and investigate in our laboratory a role for testosterone and AR in DSRCT tumor biology. The statistically significant AR-staining pattern (10/27 patients, 37%, $P = 0.0045$) in pre-treated patients is a novel finding, which supports this putative role. As a sole therapy for recurrent drug resistant disease, CAB was shown to reduce or stabilize tumor burden in an anecdotal small subset of patients for 3 to 4 months. The efficacy of this therapy in three patients was directly correlated with significant AR expression and normal testosterone levels. The failure of CAB therapy in the remaining three AR positive patients was correlated to castrate levels of testosterone which would make CAB ineffective. Criteria to be met for efficacious CAB include the presence of a positive androgen receptor ($\geq$2+) and the presence of normal testosterone levels. The need for these criteria to be met was corroborated in our in vitro models. First, we confirmed the presence of IHC positive AR status by Western blot in two patients; then demonstrated the functional status of the AR by stimulation of basal in vitro growth by supplemental DHT and finally, inhibition of basal and DHT stimulated growth by flutamide. Interestingly, in two patients (#12 and #15) the expression of AR was eventually lost in IHC and Western blot tests, which temporally corresponded to the clinical signs of relapse while on CAB. This suggested that DSRCT may up or downregulate AR possibly for survival purposes as a growth factor receptor.

The short duration of response and stability in this small cohort of end stage, heavily pre-treated DSRCT patients suggests that CAB, even in the presence of AR and normal testosterone has, at best, minor to modest activity. However, the poor performance status of these patients (all ECOG 3) most likely dampens its true effect, which may be underestimated in this small group. Our results suggest the necessity for larger clinical trials to examine CAB as a monotherapy or with chemotherapy for less heavily pre-treated, better performance status patients. This is the first report of the presence of AR expression in this disease, as it was not previously studied in any prior IHC series involving DSRCT patients [1, 10, 18, 20]. Whether the AR, as expressed in our heavily pre-treated patients, is also expressed in chemo-naïve patients remains to be answered. In addition, our results suggest the need to investigate other mesenchymal tumor types, such as gastrointestinal stromal tumors (GIST) and Ewing's, for the presence of AR, especially in male patients.

Our study also found a statistically significant positive c-Kit receptor-staining pattern (35%, $P = 0.018$). The c-Kit receptor (CD117) is a proto-oncogene that encodes for a transmembrane tyrosine kinase receptor. It has been detected in CML and in solid mesenchymal

tumors that are within the differential diagnosis for DSRCT including GIST and Ewing's sarcoma [10, 18]. An inhibitor of mutated c-Kit and related tyrosine kinase inhibitors, ST1571 (Imatinib mesylate/Gleevec), offers the possibility of a specific treatment for these tumors at a molecular level, as shown in monotherapy phase II and III trials for GIST tumors [24–26]. However, a weakness of our study is that we utilized an IHC antibody to c-Kit which detects all c-Kit forms, both wild-type and mutant. The mutated c-Kit conformation is more responsive to Imatinib than tumors with wild type c-Kit.

Prior to our study, c-Kit staining studies in DSRCT samples had shown either absent or negligible positive staining [10, 18]. One possible explanation for our 35% positive rate could be explained by the fact that the great majority of samples tested in these past studies (>80%) were obtained earlier, at diagnosis from chemo-naive, untreated DSRCT patients [10, 18]. All of our patient samples were obtained later in their clinical course after failing at least 2 chemotherapy regimens ± ASCT. Thus, wild type or mutant c-Kit expression may increase in these tumors for survival advantages after aggressive chemotherapies. This is further suggested by new c-Kit expression in two patient samples (patients # 15 and #25). One patient, #15, became c-Kit positive at 3 months after the initiation of CAB. In this patient, who originally responded to CAB for 3 months with a PR (+3 AR, normal testosterone levels), their relapsed ascites showed IHC loss of AR and new c-Kit expression (3+). In the second patient, with baseline negative AR and c-KIT status (patient #25), c-Kit was detected after three cycles of P6 and six cycles of ifosfamide and etoposide without CAB therapy. The patients' tumor never became AR positive. The identification of new c-Kit staining in two patients after treatment with either CAB or chemotherapy suggests the need to investigate the clinical use of Imatinib as a therapy for heavily-treated DSRCT patients. It would be preferable to assess c-Kit status with an antibody which only recognizes mutant c-Kit or identification of the mutant c-Kit gene by DNA sequencing studies.

Growth factor and drug resistance-associated protein status

We found negative and/or weak staining patterns for many growth factor receptors including ER, PR, EGFr, and Her-2-neu. Prior studies of these receptors reported only a single case of positive staining for Her-2-neu at initial diagnosis of untreated DSRCT patients [10]. Our study reports the second and third cases of Her-2-neu expression in heavily pretreated DSRCT, which confirms the rare expression of this marker and may explain the limited efficacy of herceptin in the treatment of DSRCT. The therapeutic significance of this rare finding suggests the need for further investigation of herceptin in IHC Her-2-neu positive DSRCT patients, especially in heavily pre-treated patients [27].

While based on a small sample, our finding of positive staining for drug resistance-associated proteins reflect the possible potential clinical utility for multiple marker identification by IHC for relapsed and pre-treated DSRCT patients. It also suggests the potential utility for marker directed modifications in chemotherapy regimens for relapsed, pre-treated DSRCT patients. High levels of GST-pi expression in five of nine patients likely reflect resistance to classical and non-classical alkylating agents, such as the platinums, that has already been detected in other tumors [28–31]. GST-pi conjugates platinum compounds to glutathione which can quench their free radical state and then allow transport out of the cell via MRP. Positive staining for MRP, already found in mesenchymal tumors homologous to DSRCT like GIST [32], and TS, commonly found in GI cancers [33], suggests acquired drug resistance in DSRCT patients to platinum agents and fluoropyrimidines, respectively [34]. This was further suggested by four out of six AR positive patients who were 3+ positive in IHC for TS expression. Interestingly, the DSRCT patients with TS overexpression were not previously treated with fluoropyrimidines in their chemotherapy regimens.

Conclusion

The IHC expression of AR (37%; 10/27) in heavily pre-treated DSRCT patients may form the clinical rationale for adjuvant treatment with CAB once maximal clinical response has been achieved by chemotherapy, radiotherapy, and surgery. Alternatively, the addition of CAB therapy based upon IHC data for AR may be incorporated into treatment regimens for patients with relapsed, pre-treated DSRCT. The IHC data showing a 35% positivity rate for c-Kit (7/20) is interesting, but whether this can translate into a response to Imatinib is unknown. This needs further study assessing the incidence of mutant c-Kit receptor status in DSRCT before any conclusions can be made. All of these efforts could yield prognostic improvements in an aggressive and highly fatal disease of young patients.

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