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EF5 BINDING AND CLINICAL OUTCOME IN HUMAN SOFT TISSUE SARCOMAS

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Purpose: To study the 2-nitroimidazole agent EF5 as a surrogate for measuring hypoxia in a series of patients with soft tissue sarcomas, and to determine whether hypoxia measured with this technique was associated with patient outcome.

Methods and Materials: Patients with soft tissue sarcomas of the head and neck, extremity, trunk, or retroperitoneum for whom surgical excision was the initial treatment of choice, were given 21 mg/kg EF5 24–48 hours before surgery. Biopsy specimens were stained for EF5 binding with fluorescence-labeled monoclonal antibodies, and the images were analyzed quantitatively. Endpoints included the relationship between EF5 binding, clinically important prognostic factors, and patient outcome.

Results: Two patients with recurrent and 14 patients with *de novo* sarcomas were studied. There were seven low-grade, one intermediate-grade, and eight high-grade tumors. No relationship was found between EF5 binding and patient age, sex, hemoglobin level, or tumor size. In *de novo* tumors, the presence of mitoses and histologic grade were positively correlated with hypoxia. High-grade and -stage *de novo* tumors had higher levels of EF5 binding compared with low-grade and -stage tumors. Patients with *de novo* tumors containing moderate to severe hypoxia (≥20% EF5 binding), high grade, or ≥7% mitoses were more likely to develop metastases.

Conclusions: Further studies in a larger cohort of patients are necessary to determine whether hypoxia, as measured by EF5 binding, is an independent prognostic factor for outcome in high-grade sarcomas. Such data should be useful to identify high-risk patients for clinical trials to determine whether early chemotherapy will influence the occurrence of metastasis. © 2006 Elsevier Inc.

EF5, Hypoxia, Sarcoma, Metastasis.

INTRODUCTION

Soft tissue sarcomas are relatively rare tumors of mesenchymal origin. In 2001, 8,000 new cases were identified in the United States, with a mortality rate of 50% (1). In the last decade, the use of limb-sparing surgery (2) with radiotherapy (3, 4) has tremendously improved local tumor control and patient quality of life. The dominant cause of death from this disease remains metastasis, especially in patients with extremity or truncal tumors (5). However, at this time it is not possible to predict which patients will develop metastases, and therefore the use of aggressive, potentially toxic chemotherapy immediately postoperatively has been limited. The ability to preoperatively identify the subgroup of patients at risk for distant failure would be extremely important in enabling the testing of new agents for efficacy in this disease.

Previous studies using the Eppendorf needle electrode have shown that pretreatment oxygenation is a prognostic marker for metastasis in this disease (6, 7). These observations infer that preoperative cellular oxygenation could be an indicator of sarcoma tumor cell aggression. Both of the previously reported studies were small and required a technically demanding and expensive piece of equipment in the operating room. Thus, despite this important observation, preoperative use of oxygen measurement has not come into use for the identification of at-risk sarcoma patients.

We have developed a clinically relevant technique for measuring hypoxia in human tumors. This technique involves the administration of the 2-nitroimidazole drug EF5 before surgery, with subsequent assessment of tissue with immunohistochemical techniques. In cells, EF5 is biochemically reduced under hypoxic conditions at a rate that is

inversely proportional to the oxygen partial pressure (pO_2) (8). The reduction product binds to protein sulfhydryls in the cell, forming adducts that can be detected with immunohistochemical techniques (9–12). EF5 has been found to be nontoxic to humans at clinically useful doses (13), has simple pharmacokinetics (13), and can detect hypoxia in a variety of human tumors (9–12). EF5 also has been formulated in a radioactive form, [18-F]-EF5 for positron emission tomographic imaging, allowing noninvasive hypoxia imaging (14–16).

The current study was performed to determine whether the Eppendorf-based results of Brizel *et al.* (6) and Nordsmark *et al.* (7) could be replicated with EF5 binding, given that our previous studies have shown that EF5 binding in soft tissue sarcomas correlates with Eppendorf needle electrode values (11). We hypothesized that (*1*) low-grade tumors would have low EF5 binding and high-grade tumors would have higher levels of binding, and that (*2*) patients who developed metastases would have a higher level of EF5 binding than those who did not. Both of these hypotheses were supported.

METHODS AND MATERIALS

Human subjects

Written, informed consent, approved by the University of Pennsylvania Institutional Review Board, the Clinical Trials and Scientific Monitoring Committee of the University of Pennsylvania Cancer Center, the Cancer Therapeutics Evaluation Program, and the National Cancer Institute was obtained from all patients entered on this study.

EF5 studies

Drug administration. The National Cancer Institute, Division of Cancer Treatment supplied EF5 in 100-mL vials containing 3 mg/mL EF5 and 5% dextrose in water with 2.4% alcohol. The drug solution was administered intravenously with a peripheral catheter at a rate of approximately 350 mL/h to a total dose of 21 mg/kg, on the basis of a previously conducted phase I trial (13).

Tissue acquisition, staining, and analysis. All patients studied herein were scheduled for therapeutic surgical removal of a sarcoma. Approximately 24 h (range, 18–30 h) after completion of drug administration, the tumor was surgically resected. Sterile tumor tissue was placed in iced EXCELL 610 media (JRH Biosciences, Lenexa, KS) with 15% fetal calf serum. These tissues were immediately processed to determine "*in situ* EF5 binding" and "cube reference binding," as previously described (8, 9, 11, 12). The final EF5 absolute intensity values were calculated after corrections for lamp intensity, tissue section thickness, camera exposure time (8, 9, 11, 12), and EF5 drug exposure (13). The final reported value, "percent cube reference binding" was calculated by dividing the corrected *in situ* EF5 binding intensity by the cube reference binding value and multiplying this value by 100.

At least four sections from each tumor specimen, separated by at least 0.5 mm in the "z" direction of sectioning, were examined (17). The presence of viable tumor tissue was confirmed on all tissue sections. The use of a calibrated fluorescence scale and a measure of the maximum possible binding of each tumor (cube reference binding) allowed a pixel-by-pixel analysis of the ob-

served EF5 binding as a percentage of cube reference binding. Thus, the endpoints for EF5 binding presented herein reflect both the level and the area of binding (12, 18, 19). Data were summarized by providing a cumulative frequency analysis of all pixels. Selected points in the cumulative frequency curve were denoted by $CF_{\#\#}$. Thus, $CF_{95} = 20$ would mean that 95% of the EF5 values in the image were at or below 20% of cube reference binding. Median EF5 binding would be denoted as CF_{50} (12).

We have previously described our procedure to produce oxygen maps from the EF5 binding images (8, 12). Briefly, a pixel-by-pixel analysis of percent cube reference binding is performed. This analysis is displayed as a two-dimensional map of EF5 binding. With extensive data from *in vitro* studies (8), it is possible to convert absolute EF5 binding into tissue pO_2. On the basis of the EF5 binding values we have denoted a five-range map, in which cells with physiologic oxygen levels ($\geq$10% oxygen) have approximately 1% EF5 binding, modestly hypoxic cells (approximately 2.5% oxygen) have approximately 3% EF5 binding, moderately hypoxic cell (approximately 0.5% oxygen) have approximately 10% EF5 binding, severely hypoxic cells (approximately 0.1% oxygen) have approximately 30% EF5 binding, and anoxic cells (0% oxygen) have 100% EF5 binding. These regions can then be color coded and presented as an oxygen map.

Histopathologic grading of malignancy and microscopic necrosis

All tissue sections were reviewed by a pathologist (P.Z.) at the Hospital of the University of Pennsylvania to assure that binding occurred in viable tumor tissue and to confirm histopathologic diagnosis and tumor staging. The pathologist had no knowledge of the EF5 binding results. The number of mitoses was counted in 50 high-power fields. The extent of microscopic necrosis was analyzed on a continuous scale of 0–100% by examining all sections of the tumor taken for histologic diagnosis.

Statistical analyses

Patient characteristics were summarized with descriptive statistics. The mean, standard deviation (SD), and median were calculated for continuous variables. For discrete data, frequency tables and proportions were used. Time to local recurrence and time to metastasis were defined as the time of administration of EF5 to that of radiographically confirmed local recurrence or metastasis, respectively. Disease-specific survival time was the time from administration of EF5 to date of death due to disease. Subjects who had not experienced the event of interest were censored at their last follow-up date. For analyses of EF5 binding, we used two endpoints: the maximum CF_{95} (CF95_Max) and the average CF_{95} (CF95_Avg). The CF95_Avg was based on examination of at least four tissue sections (12, 20). Because of the small number of subjects, the relationship between EF5 binding and tumor-related factors were evaluated by nonparametric tests, including Wilcoxon rank–sum and Kruskal-Wallis rank tests. Time to event data were summarized by Kaplan-Meier methods and compared by log–rank tests.

RESULTS

Between May 1998 and March 2004, 17 patients with non-intraperitoneal spindle cell tumors agreed to participate in the EF5 study and received the drug intravenously 24–48 h before surgery. A recurrent tumor was suspected in 1 patient on the basis of imaging, but a mass could not be found at the

time of surgery, and the biopsy from the region was scar tissue. In the remaining 16 patients, tumor was removed and analyzed for EF5 binding. Five female and 11 male patients with a median age of 54 years (range, 30–74 years) were studied. The tumor characteristics, adjuvant therapy, and outcome for these 16 patients are presented in Table 1. The majority of tumors were located in the extremities, and their maximum diameter, according to measurements made in the pathology laboratory, ranged from 1.7 to 20 cm. Eight tumors were high grade and eight were low or intermediate grade.

The two endpoints for EF5 binding considered for analyses, the CF95_Avg and the CF95_Max, were highly correlated to each other. Spearman's rank correlation was 0.88 and 1.00 for *de novo* and recurrent tumors, respectively (data not shown). Therefore, all further analyses were based on CF95_Max.

Figures 1 and 2 show the EF5 binding patterns in a low- and high-grade sarcoma, respectively. Figure 1 shows the binding level in a *de novo*, low-grade myxofibrosarcoma/ malignant fibrous histiocytoma of the thigh. This tissue was sparsely cellular with widely spaced nuclei and extensive myxomatous matrix. Very little EF5 binding was present (Fig. 1a). The oxygen map presented in Fig. 1c confirms that all pO$_2$ values were in the physiologic range, represented by green regions. The regions of tumor that contain extensive matrix are presented in black because there was no EF5 binding in these regions. This would be expected because cellular metabolism is required for the reduction and binding of the agent. In contrast, Fig. 2 shows the EF5 binding in a high-grade pleomorphic sarcoma of the chest wall. This tumor was poorly differentiated and cellular. The oxygen map demonstrates that substantial hypoxia was present; these areas are represented by regions of red and orange in the oxygen map (Fig. 2c).

We have analyzed the relationships between EF5 binding, patient factors, and tumor-related factors. There was no evidence in our data to support a significant correlation between EF5 binding (either CF95_Avg or CF95_Max) and age, sex, tumor size, or hemoglobin level (data not shown). However, in *de novo* tumors, there was a statistically significant relationship between CF95_Max and the presence of mitoses ($p = 0.001$) and histologic grade ($p = 0.003$).

For the purpose of outcome analysis, the patients were divided into those that, at the time of EF5 administration, had recurrent tumors (e.g., previously treated, Table 1) vs. *de novo* tumors. (We use the term *recurrent* to refer to those patients ($n = 2$) who were given EF5 for tumors that had been previously treated by surgery or other means. We use the term *locally recurrent* to refer to the situation in which the tumor returns in the same region after the surgery when EF5 was given. In our series, none of the recurrent patients developed local recurrence, but one patient developed metastasis. However, 3 of the *de novo* patients developed local recurrence.) There were 2 patients with recurrent tumors, 1 with a high-grade lesion, and 1 with a low-grade lesion. One of these tumors was of the extremity, and 1 was truncal. The

Table 1. Tumor characteristics, adjuvant therapy, and patient outcome

Variable	
n	16
Tumor histology	
Fibro/myofibroblastic tumor	7
Leiomyosarcoma	3
Liposarcoma	3
Others	3*
Tumor site	
Extremity	9
Truncal	5
Other (head/neck; retroperitoneal)	2
Tumor grade	
High	8
Low/intermediate	8
Recurrent vs. *de novo*	
Recurrent	2
De novo	14
Adjuvant therapy (*de novo* patients)†	
Pre-op radiation	3
Pre-op chemotherapy and radiation	1
Post-op radiation	6
Post-op chemotherapy	1
Post-op chemotherapy and radiation	1
Previous therapy (recurrent patients)	
Radiation and chemotherapy	1
Adjuvant therapy (recurrent patients)	
Post-op radiation	1‡
Follow-up (mo)	
Mean ± SD	22 ± 21.5
Median	13.5
Range	2.8–69.4
Metastasis	
No	10
Yes	6
Local recurrence	
No	13
Yes	3
Death	
No	11
Yes	5

Abbreviations: Pre-op = preoperative; Post-op = postoperative; SD = standard deviation.

Values are *n* unless otherwise indicated.

* *n* = 1 each for synovial sarcoma, sarcoma not otherwise specified, atypical nerve sheath tumor.

† Refers to planned treatment immediately following surgical removal. Two patients additionally received adjuvant therapy following diagnosis of local recurrence (radiation) or metastasis (chemotherapy).

‡ Patient received two radiation treatments and refused further treatment.

CF95_Avg and CF95_Max binding were found to be <4% in both of these recurrent tumor (i.e., the tumors were oxic/modestly hypoxic). The patient with the high-grade tumor developed metastases at 21.1 months after EF5 administration and died as a result of her disease. The patient with the recurrent low-grade tumor was alive and without further recurrence at the time of analysis.

In contrast to the recurrent tumors, the *de novo* tumors had considerable intra- and intertumoral hypoxic heteroge-
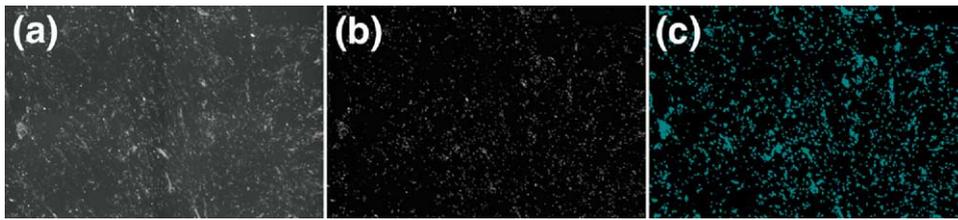
Fig. 1. Images of EF5 binding in Patient 46, who had a myxofibrosarcoma/malignant fibrous histiocytoma of the thigh. (a) EF5 binding; (b) same section counterstained with Hoescht 33342, to show the location of the nuclei; (c) resultant oxygen map based on EF5 binding. Green/blue = ≥10% oxygen.

neity (Fig. 3). Low- and intermediate-grade *de novo* tumors were oxic or modestly hypoxic, with a mean CF95_Max of 2.6% (SD = 1.9%). At the time of data analysis, 1 patient with a low-grade tumor had developed a local recurrence. None of the patients with low- or intermediate-grade tumors had developed metastases, and all were alive at 2.8–69.4 months of follow-up. In contrast, most of the high-grade tumors contained regions of moderate to severe hypoxia, with a mean CF95_Max of 43.8% (SD = 41.2%) (Fig. 3). Six patients had developed metastasis, 3 patients had developed local recurrence, and 5 patients had died of their disease (Table 1).

Survival analysis was performed on patients with *de novo* tumors. As discussed above, data were sorted according to CF95_Max. Rather than simply dividing the patients into groups in which EF5 binding was above vs. below the median, we analyzed them on the basis of the observation that there were two very different groups of EF5 values. Eight patients had CF95 values <7%, and the remaining 6 patients had values >20%. The value of 20% EF5 binding corresponds to moderate to severe hypoxia, as we have previously reported (20). Thus, patients with CF95_Max <20% were compared with patients with CF95_Max ≥20%. Clinical outcomes considered were local recurrence, metastasis, and disease-specific survival. Considering all 14 patients with *de novo* tumors, we found that the development of metastases was significantly associated with oxygen status

(p = 0.05) (Fig. 4), but survival (p > 0.05) and local recurrence (p > 0.05) were not (data not shown). The mean CF95_Max binding was significantly higher (p = 0.04) among those who had metastases (n = 5, median = 32.7%) than among those who did not (n = 9, median = 2.8%).

The prognostic significance of necrosis, grade, stage, and mitosis in *de novo* tumors was considered independently. Neither tumor stage nor the presence (n = 6) or absence of necrosis (n = 6) was significantly correlated with the any outcome measure. However, the level of mitosis (≤7% vs. >7%; p = 0.05) and tumor grade (high vs. intermediate/low; p = 0.03) were significantly associated with time to metastasis (p < 0.01).

DISCUSSION

Soft tissue sarcomas are a relatively rare and heterogeneous group of tumors. For both of these reasons there is debate as to the most important clinical/pathologic staging factors. At least nine pathologic staging systems have been reported in the literature (21). The importance of accurate staging of these tumors lies in the realization that initial tumor control is critical, given that a majority of patients who experience distant failure usually die of their disease (22). Because all the current staging systems rely only on anatomic and histologic parameters, the addition of molecular and biologic
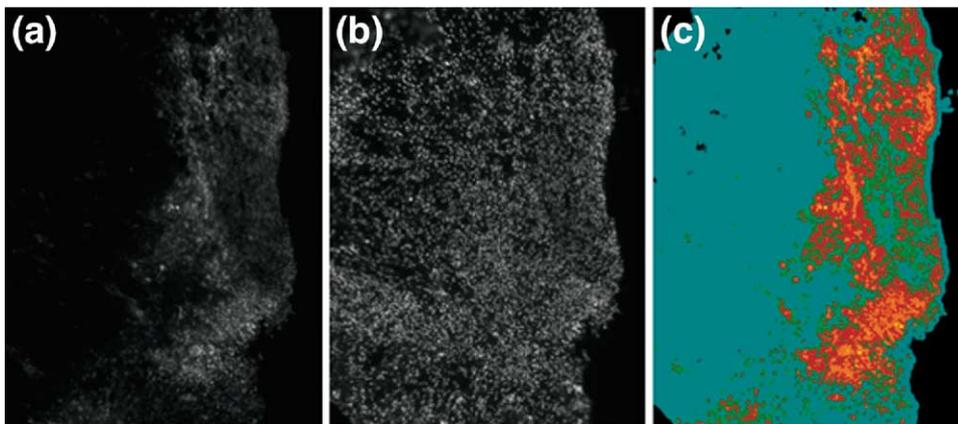

Fig. 2. Images of EF5 binding in Patient 89, who had a high-grade pleomorphic sarcoma of the chest wall. (a) EF5 binding; (b) same section counterstained with Hoescht 33342, to show the location of the nuclei; (c) resultant oxygen map based on EF5 binding. Green/blue = ≥10% oxygen; red/orange = 0.5–2.5% oxygen; orange/yellow/white = <0.5% oxygen.
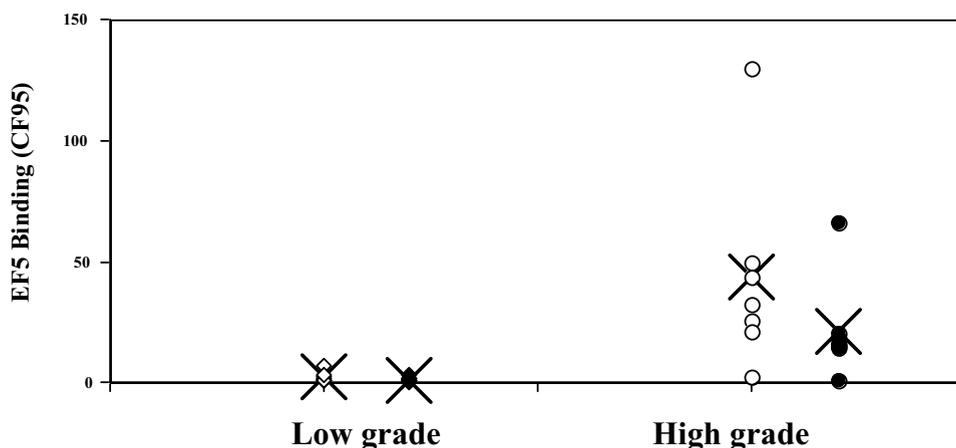
Fig. 3. EF5 binding in sarcomas. The tumors were grouped on the basis of high grade (circles) vs. low grade (diamonds). The endpoints, CF95_Max (open symbols) and CF95_Avg (closed symbols) are shown. For each group, the cross denotes the mean value for the group.

parameters could improve the predictive model (23). The data presented herein add to that information, especially because it is now understood that many molecular and biologic changes are influenced by the tumor microenvironment (24).

In two previously published studies performed with the Eppendorf needle electrode, hypoxic sarcomas were shown to be more likely to metastasize than oxic sarcomas (6, 7). The underlying biology of this phenomenon has been attributed to the effect of hypoxia on genetic, molecular, and cytokine-induced changes in tumor biology. These observations might have considerable clinical significance regarding therapeutic interventions. Interestingly, the use of hypoxia-driven therapies is likely to be ineffective in this disease. The prometastatic genetic changes that are thought to occur as a result of hypoxia in sarcomas are likely already in play by the time the tumor is resected. Yet, the early, prospective
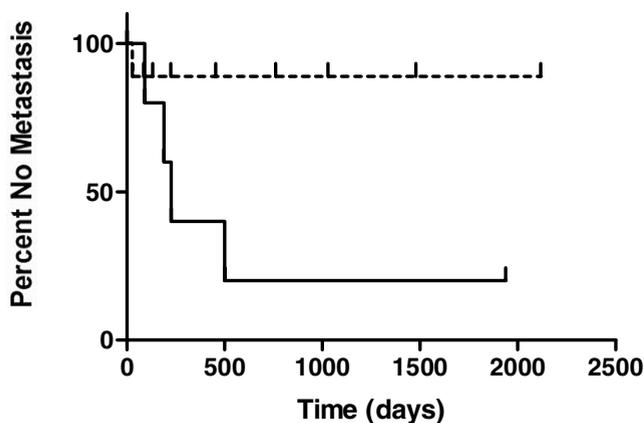

Fig. 4. Fourteen patients with *de novo* sarcomas were followed for the occurrence of metastasis. Patients with tumors in which the maximal EF5 binding, as measured by CF95_Max (see text for details), was ≥20% of cube reference binding (solid line) were found to have a more rapid time to metastasis compared with patients whose tumors had >20% of cube reference binding (broken line). Twenty percent or greater of cube reference binding approximately represents tissue pO_2 levels at or below 0.2%.

identification of patients at highest risk for metastases could allow early treatment that might have an impact on the natural history of the disease. Because several chemotherapy agents, including doxorubicin and ifosfamide, are active in this disease (25), early determination of which patients are most likely to develop metastases could be helpful to optimize therapy and/or identify more effective agents.

However, for clinical utility, the technique for hypoxia measurement must be easily applied. The Eppendorf electrode and other newly developed needle electrode–based techniques are limited in their application, mainly because of the equipment and technical expertise required to obtain accurate results. In contrast, immunohistochemical studies are commonly used for diagnosis and prognosis in many diseases today, and surgical excision is the treatment of choice for soft tissue sarcomas. EF5 is safe and well tolerated (9, 13). Further simplification might be possible once this agent is tested in humans with the positron emission tomography–imageable agent, [18-F]-EF5. Preliminary data from rodent studies and early images in human brain tumors are encouraging (16).

Our data support and extend previous literature regarding prognostic factors in sarcomas. Tumor grade is considered to be the most reliable prognostic endpoint in this disease (21), and it was identified to be prognostic in our group of patients. As found in most other tumors sites, with the possible exception of breast cancer (26, 27), tumor size was found to be neither prognostic nor correlated with pO_2 in our series. The presence of hypoxia in regions containing necrosis is similar to what we reported regarding human gliomas (12, 20). However, the extent of necrosis was not found to be of prognostic value. Our data do not associate pretreatment hemoglobin with the level of hypoxia, which is similar to the 1996 findings of Nordsmark *et al.* (28). This is in contrast to the association of hypoxia with low hemoglobin and poor outcome in both head-and-neck and cervical cancers (29, 30). We found a positive relationship between the presence of mitoses in tumors and the level of

hypoxia. In addition, the extent of mitosis was significant for outcome (metastasis) in this group of patients. Nordsmark *et al.* found no correlation between tumor oxygenation and labeling index, as measured by iododeoxyuridine incorporation, but did report a significant correlation between the median pO_2 and the tumor cell potential doubling time. The importance of cell proliferation, especially in the context of tumor hypoxia, is an important topic for further study.

Similar to our findings in brain tumors (12, 20), low-grade sarcomas were found to have minimal EF5 binding and were therefore judged to be at physiologic oxygen levels. We have found hypoxia to be a heterogeneous characteristic of high-grade *de novo* tumors, both in sarcomas and in brain tumors (12, 20).

In summary, the present data and previous reports provide evidence that hypoxia can be used to identify patients with *de novo* sarcomas at high risk for metastases. Studies of a larger number of patients are required to determine whether hypoxia is an independent prognostic factor. However, with the availability of clinically relevant hypoxia detection procedures, studies of early intervention to prevent or delay metastases can proceed.

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