Impact of Heterogeneity in the Predictive Value of Kinetic Parameters in Canine Osteosarcoma

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ABSTRACT

Intratumoral heterogeneity has been identified as a potential problem in the efficacy of predictive assays. Canine osteosarcoma is an extremely heterogeneous solid tumor that has been shown to be an excellent model for the human disease. Intratumoral heterogeneity of kinetic parameters and the effect of heterogeneity on predicting outcome of treatment (time until metastasis) were studied in dogs with naturally occurring osteosarcoma. Dogs were treated with amputation or tumor excision and limb-sparing followed by chemotherapy with cisplatin. Kinetic parameters evaluated included v, duration of DNA synthesis (T_S), and potential doubling time (T_{pot}), determined using in vivo labeling with bromodeoxyuridine and flow cytometry.

In 30 tumors, multiple samples were obtained and evaluated. There was significantly more variation between tumors from different dogs than intratumoral variation of v, T_S, and T_{pot}. Variations in v, T_S, and T_{pot} within a tumor were associated with both sample location and tumor subpopulation. Time to metastasis was determined in 51 dogs with tumors sampled for kinetics. Multiple samples were available from 25 of these tumors. Cox proportional hazard analysis was performed using either the fastest or slowest T_{pot} from each sample. The fastest available T_{pot}s were highly significant (P < 0.001) for prediction of outcome. The slowest available T_{pot}s were also significant predictors, although the statistical strength was compromised (P = 0.024). Obtaining at least two samples in large tumors known to be heterogeneous is recommended to improve the predictive ability of T_{pot}. v is a more limited predictor but can be useful when T_{pot} is not available. In canine osteosarcoma, an extremely heterogeneous tumor, kinetic parameters were shown to be predictors of outcome.

INTRODUCTION

Kinetic parameters including LI or v (a dimensionless function related to labelling index that accounts for division of labelled cells; Ref. 1), duration of DNA synthesis (T_S), and potential doubling time (T_{pot}) are being investigated as predictors of treatment response for both radiation and chemotherapy (2—5). Intratumoral heterogeneity has been identified as a potential problem in the efficacy of predictive assays in general (6). Efforts have been made to define the heterogeneity of kinetic parameters. Begg et al. (7) evaluated the intratumoral sampling variations of kinetic parameters in six transitional cell carcinomas of the bladder and seven head and neck squamous cell carcinomas. Using the coefficient of variation as a measure of intra-tumoral variation of T_S, T_{pot}, and v. The parameters were evaluated for any predictive significance for the end points of time to metastasis and survival. When possible, multiple samples from individual tumors were evaluated. Intratumoral heterogeneity and the impact of that heterogeneity on the predictive significance of the assays were studied.

MATERIALS AND METHODS

Patient Population. Client-owned dogs admitted to the Colorado State University Veterinary Teaching Hospital with naturally occurring osteosarcoma were used for this study. All dogs were free of gross metastasis and underwent treatment for the primary tumor by either amputation or tumor excision, often associated with a limb-sparing procedure. Some dogs had a cisplatin-impregnated polymer sponge (OPLA-Pt) implanted in the tumor bed to assess its effect on local tumor control. All dogs received from 1 to 6 doses of i.v. cisplatin at 70 mg/m². Patients were reevaluated following treatment at 1, 2, and 3 months, and every three months thereafter or if any clinical abnormalities developed. Reevaluation routinely included thoracic radiographs to evaluate for metastatic disease. Other diagnostic procedures, such as nuclear bone scanning or radiographic bone surveys, were performed if bony metastases were suspected. All procedures performed on these dogs were approved by the Colorado State University Animal Care and Use committee.

Sample Procurement. At a known time prior to sampling, 300 mg BrdUrd diluted in 60 ml sterile saline was injected i.v. Samples were obtained at the time of tumor excision, generally from 3 to 6 h after BrdUrd administration. After the tumor was excised, it was bisected longitudinally with a band saw.

Naturally occurring canine osteosarcoma is a unique and well-documented model for the study of local and systemic cancer control in solid tumors (10, 11). Canine osteosarcoma is an extremely heterogeneous tumor. Tumor heterogeneity is apparent not only between different tumors but often within the same tumor. Gross, radiological, histological, and DNA index intratumoral heterogeneity have been reported (11—13). The biological behavior of the disease is predictable and well documented. If the primary tumor is removed and no adjuvant chemotherapy is administered, 90% of dogs will die of metastatic disease within 1 year (14, 15). With the addition of chemotherapy, generally cisplatin, the onset of metastatic disease can be delayed (16—18).

In this study, kinetic parameters of canine osteosarcoma were determined prior to treatment of the primary tumor. The parameters evaluated included T_S, T_{pot}, and v. The parameters were evaluated for any predictive significance for the end points of time to metastasis and survival. When possible, multiple samples from individual tumors were evaluated. Intratumoral heterogeneity and the impact of that heterogeneity on the predictive significance of the assays were studied.

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2 To whom requests for reprints should be addressed, at Department of Radiological Health Sciences, Colorado State University, 300 W. Drake, Fort Collins, CO 80523.
3 The abbreviations used are: LI, labeling index; v, a dimensionless quantity related to LI; T_{pot}, potential doubling time; T_S, duration on DNA synthesis; BrdUrd, bromodeoxyuridine; PBS, phosphate-buffered saline; DI, DNA index.

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When tumor specimens were larger than 1 cm³, multiple samples were obtained for intratumoral heterogeneity evaluations. The appearance of the region and the topographic location within the tumor were recorded for each sample. Samples were divided, and a representative portion of each was placed in 10% neutral buffered formalin for histological processing and evaluation. When necessary, samples were decalcified in formic acid with an ion-exchange resin, dehydrated, and then embedded in paraffin. Six to seven 7 μm sections were cut, mounted on slides, and stained with hematoxylin and eosin. The remainder of each sample was processed for flow cytometric evaluation.

Cell Staining. Single cell suspensions for flow cytometric evaluation were obtained by coarsely mincing the tumor sample with a scalpel blade and then mechanically disaggregating the sample in PBS with a Stomacher (VWR, Denver, CO) as described previously (12). Following filtration, cells were counted, aliquoted, and fixed in 50% ethanol in citric acid-buffered saline (19). The tubes were then stored at 7°C until staining, and flow cytometric analysis was done. The previously fixed cells were stained using a modification of a previously published technique (20). Briefly, after washing in PBS, cells were resuspended in 0.2 mg/ml pepin (21) in 2 × HCl in PBS and incubated for 20 min at 37°C. The HCl allowed partial DNA denaturation to occur. and the simultaneous addition of pepsin decreased debris and enhanced staining to the resultant nuclei (21). Cells were washed twice in 0.5% Tween (Sigma Chemical Co., St. Louis, MO) in PBS. The pellet was resuspended in 500 μl of crystalline 2% bovine serum albumin (Sigma Chemical Co.) in PBS and incubated for 20 min at room temperature in the dark. Mouse-derived monoclonal antibody against BrdUrd (Becton Dickinson, San Jose, CA) was added, and the cells were incubated for 45 min at room temperature. Cells were then washed in 0.5% Tween in PBS. Fluorescein-conjugated F(ab')2 goat anti-mouse IgG (AMAC, Inc., Westbrook, ME or Cappel, Durham, NC) was added, and cells were incubated at room temperature for at least 20 min before flow cytometric analysis. Stained samples were stored at 7°C overnight prior to flow cytometry.

When possible, tumor cells were prepared for independent DI determination. Lymphocytes from the tumor-bearing dog or a normal donor dog were harvested by centrifuging a blood sample obtained through Histopaque (Sigma) and then by using histomorphometry. Samples of adjacent normal or reactive tissue structures were excluded from analysis. In some tumors, fewer cells were available for analysis. The histograms were analyzed on a personal computer using MultiCycle software and Multi2D software (Phoenix Flow Systems, San Diego, CA). Relative movement of green fluorescent cells was determined as described by Begg (20). The kinetic parameters of primary osteosarcoma in patients undergoing treatment were correlated with outcome using Kaplan-Meier analysis. Other parameters, such as DNA index and treatment groups, were also included in analyses. Differences between groups were evaluated for significance using a Cox proportional hazards evaluation.

RESULTS

A total of 57 high grade osteosarcomas were included in this study. Tumor locations included the radius (n = 15), humerus (n = 13), tibia (n = 9), femur (n = 9), ulna (n = 4), and mandible (n = 2) and 1 each in the occiput, vertebrae body, rib, scapula, and nasal cavity. These dogs were part of larger ongoing studies evaluating a variety of treatment regimens for osteosarcoma (17, 18). From these 57 tumors, 135 samples met criteria for DI and kinetic parameter analysis. Of the 57 tumors, 40 had at least one aneuploid population, while in 17 tumors, only diploid tumor populations were identified. Of the 135 samples, 42 were diploid and 93 were aneuploid. The mean percentage of tumor of the histomorphometric correlate for diploid samples was 96.4. Unpaired t tests were performed on diploid versus aneuploid tumors populations for all kinetic parameters (Table 1). T50 was significantly longer in aneuploid than in diploid tumors (P = 0.0184). v and Tpot were not significantly different.

In 30 osteosarcomas, v was successfully evaluated from at least two locations within a tumor. Using a general linear model, it was determined that variation between tumors from different dogs was significantly greater than the variation within tumors (P = 0.0077; Fig. 1). In 26 primary tumors, T50 and Tpot were successfully evaluated from at least 2 sites, and variation was significantly greater between tumors from different dogs than within a tumor (P = 0.0025 and P = 0.0001, respectively; Fig. 1). The variation in kinetic parameters within individual tumors was analyzed using a general linear model, based on location of the tumor sample and tumor subpopulation. Tumor location was classified as adjacent to central necrosis, from viable-apparenting tumor within the cortex, and from viable-apparenting tumor which extended outside of the cortex. Tumor subpopulations were distinguished by the DI of the sample. Variations in v, T50 and Tpot within a tumor were associated with both sample location and tumor subpopulation (P = 0.0068, 0.0047, and 0.027, respectively). Samples adjacent to central necrosis were associated with the lowest v and lowest Tpot from that tumor (P = 0.026 and 0.024, respectively). Fifty-one dogs with high grade osteosarcomas of the extremities and scapula were evaluated for time until metastasis and survival. All of these dogs were treated with tumor excision via amputation or limb-sparing surgery and adjuvant cisplatin administered i.v. The doses of cisplatin administered varied from 1–6, although most dogs received either 2 or 4 doses. The possible dose effect of cisplatin was included in the statistical analysis. In 25 of these dogs, multiple samples were obtained from the tumor for kinetic analysis. In the remaining 26 dogs, only one sample was available, due either to the tumor being so small that it could not be divided into multiple samples.
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Fig. 1. Distribution of pretreatment cell kinetic parameters for canine osteosarcoma. Top, \( \nu \) as a percentage; middle, \( T_s \) in h; bottom, \( T_{pot} \) in days. The patient number is on the abscissa with each data point representing an individual sample from the tumor. Diploid (\( \bigcirc \)) and aneuploid (\( \bigcirc \)) are distinguished.

or that multiple samples were obtained, but only one was successfully evaluated. The mean, SD, coefficient of variation, and median values of the population using the fastest \( T_s \) and \( T_{pot} \) and largest \( \nu \) values and the slowest \( T_s \) and \( T_{pot} \) and smallest \( \nu \) values available for a given tumor were determined (Table 2). These two data sets, designated large/fast and small/slow were both used for all subsequent analyses. Using Cox proportional hazards regression analysis, selected factors were bivariately analyzed for their impact on time to metastasis (Table 3). Where appropriate, both continuous and nonparametric data was used for analysis. Nonparametric groupings were based on the statistical values (median) or on biological divisions that were apparent when \( T_{pot} \) and \( \nu \) were plotted against the time to metastasis (Fig. 2). Tumors with a \( \nu \) value of 12% and greater were associated with a short time until metastasis; therefore, this value was included in the bivariate analyses. Time to metastasis, however, generally increased as \( T_{pot} \) values increased. Multivariate analyses were also performed, and the most appropriate multivariate models were selected (Table 4). Although \( T_{pot} \) was a significant predictor in both the large/fast group \((P < 0.001)\) and the small/slow group \((P = 0.024)\), the difference is illustrated with Kaplan-Meier plots (Fig. 3). Whether a patient received 1 to 2 cisplatin administrations compared to 3 or more was also significant \((P = 0.008)\), and the relationship between treatment group and kinetics in the large/fast group is shown in Fig. 4. Time to metastasis was affected profoundly in dogs with fast \( T_{pot} \), depending on whether they received 1 or 2 cisplatin compared to 3 or more cisplatin administrations \((P = 0.025)\), whereas dogs who had slow \( T_{pot} \) did not have a significant difference in outcome, regardless of the number of cisplatin administrations.

DISCUSSION

Intratumoral heterogeneity of kinetic parameters has been documented, but the impact on the predictive potential of kinetic parameters had not previously been studied. Intratumoral variation in kinetic parameters depended on sample location and the tumor subpopulation sampled. Samples taken from the center of the tumor, which in canine osteosarcoma is often grossly necrotic, often did not produce samples that reflected the kinetics of the rest of the tumor. In samples from this central region, \( \nu \) and \( T_s \) were statistically lower and slower than other samples from within the tumor. However, kinetic heterogeneity was not solely due to sample location. Variation in kinetic parameters was also associated with the tumor subpopulation, as determined by differences in the DI. Multiple DIs within a single tumor are not uncommon in canine osteosarcoma (12). Kinetic parameters appear to be a phenotypic expression of the subpopulation. Despite these variations, the general linear model demonstrated more intertumoral than intratumoral variation of \( \nu, T_s, \) and \( T_{pot} \). Perhaps because these are tumor subpopulations and have a common origin, the kinetic parameters are also related. The fact that there is less intratumoral than intertumoral variation is of paramount importance if these kinetic parameters are to be useful predictors. From a practical standpoint, avoiding the regions adjacent to areas of necrosis is important but not the only factor in obtaining a representative sample in a heterogeneous tumor such as canine osteosarcoma. In large tumors, additional samples may reveal different kinetic parameters due to variation in tumor subpopulations.

The distribution of DIs in this study is similar to previously published reports for canine and human osteosarcoma (12, 25–27).

Table 2 Kinetic parameters from 51 dogs with tumors of the extremity, who were followed for time to metastasis

<table>
<thead>
<tr>
<th>Tumor Number</th>
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<th>Tumor Number</th>
<th>Tumor Number</th>
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<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

- \( \nu \): Mean and SD (\( h \))
- \( T_s \): Mean and SD (\( h \))
- \( T_{pot} \): Mean and SD (\( h \))

<table>
<thead>
<tr>
<th>( \nu )</th>
<th>( T_s )</th>
<th>( T_{pot} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>SD</td>
<td>CV(%)</td>
</tr>
<tr>
<td>8.7 ± 5.4</td>
<td>61.7</td>
<td>8.1</td>
</tr>
<tr>
<td>12.7 ± 6.5</td>
<td>51.1</td>
<td>10.5</td>
</tr>
<tr>
<td>6.3 ± 5.1</td>
<td>80.8</td>
<td>4.8</td>
</tr>
</tbody>
</table>

- Slowest available \( T_s \) and \( T_{pot} \) and smallest \( \nu \)

<table>
<thead>
<tr>
<th>( \nu )</th>
<th>( T_s )</th>
<th>( T_{pot} )</th>
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<tbody>
<tr>
<td>Mean</td>
<td>SD</td>
<td>CV(%)</td>
</tr>
<tr>
<td>6.9 ± 4.3</td>
<td>62.7</td>
<td>6.1</td>
</tr>
<tr>
<td>17.2 ± 11.5</td>
<td>66.7</td>
<td>14.3</td>
</tr>
<tr>
<td>8.4 ± 5.7</td>
<td>67.9</td>
<td>6.9</td>
</tr>
</tbody>
</table>

\( a \) CV, coefficient of variation.
Aneuploid tumors had significantly longer $T_{S}$, which is consistent with reports of other tumor types (4), but no difference in $v$ or $T_{pot}$ existed. It has been speculated that normal tissues included for analysis may falsely increase $T_{pot}$. Regardless, the effect of normal tissues on flow cytometrically determined tumor cell kinetics in diploid tumors must be addressed. In this study, only diploid samples that were falsely decreased the $T_{pot}$. Interestingly, in osteosarcoma the surrounding normal bone has often become reactive, which could decrease the $T_{pot}$. Regardless, the effect of normal tissues on flow cytometrically determined tumor cell kinetics in diploid tumors must be addressed. In this study, only diploid samples that were quantitated as at least 85% tumor were included in the analysis.

The predictive value of ploidy in human osteosarcoma is controversial. In this study, aneuploid tumors were not indicative of larger $v$ or shorter $T_{pot}$. Aneuploidy was also not predictive of time to metastasis in bivariate analysis (Table 3).

Cox proportional hazard analysis was performed for the end points of time to metastasis and survival. However, the results for both end points were similar, and survival analyses were excluded for the sake of brevity. The end point of time to metastasis is considered most important in canine osteosarcoma because survival is influenced by when the owner makes the decision to euthanize the dog. Dogs with tumors of the axial skeleton were excluded from Cox proportional hazard analysis because the biological behavior of tumors in these locations is known to be different from tumors of the appendicular skeleton; to evaluate a potential predictive assay, it is important to minimize confounding factors.

Cox proportional hazard analyses were performed on both the largest/fastest and small/slow samples to determine any biological significance and to study the impact of heterogeneity on the predictive value of the assays. Samples were analyzed based on these groupings because variation in samples was due to two factors, biopsy location and subpopulation. Hence, the smallest $v$ or slowest $T_{pot}$ were not always associated with the central sample or a given subpopulation. When largest/fast times available were used for regression analysis, $T_{pot}$ was still predictive but with decreased statistical power. From a biological standpoint, this implies that the largest/fast subpopulation from the primary tumor is most strongly associated with the development of metastasis. This could be because the most rapidly dividing subpopulation has characteristics that would also enhance the development of metastatic disease, or it could be purely a reflection of growth rate that both rapid and slow subpopulations metastasize but the metastases from the rapid subpopulation grow more rapidly, thus becoming clinically evident first. The difference in predictive power pending sample selection demonstrates the potential influence of tumor heterogeneity.

Models were constructed for the best predictors of treatment outcome. It was expected that the number of cisplatin courses administered would affect outcome, and this agrees with a larger study (28). It is impressive that tumor cell kinetics are such statistically strong predictors, especially when considering that the degree of tumor chemosensitivity, which was not evaluated in this study, must also impact outcome. The outcome was analyzed for both $T_{pot}$ and number of cisplatin administrations (Fig. 4). The importance of this is that a distinct subpopulation can be identified that does not benefit like the entire population from the addition of more that two chemotherapy administrations. This demonstrates that predictive assays performed

![Graph](image-url)
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Table 4 Final multivariate models for time to metastasis

<table>
<thead>
<tr>
<th>Factor</th>
<th>Hazard ratio</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-2 cisplatin vs. more</td>
<td>0.022/0.050</td>
<td>0.425/0.497</td>
</tr>
<tr>
<td>$T_{pot}$ = 4.7 vs. faster</td>
<td>0.002/0.054</td>
<td>3.357/2.032</td>
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Likelihood ratio statistic

On 2 DF for fastest $T_{pot}$ = 15.028; $P < 0.001$
On 2 DF for slowest $T_{pot}$ = 8.134; $P = 0.017$

If $v$ but not $T_{pot}$ is available, largest vs smallest $v$ values

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>1-2 cisplatin vs. more</td>
<td>0.004/0.006</td>
<td>0.367/0.391</td>
</tr>
<tr>
<td>$v$ ≤ 4.7% vs. more</td>
<td>&lt;0.001/&lt;0.001</td>
<td>4.399/5.258</td>
</tr>
</tbody>
</table>

Likelihood ratio statistic

On 2 DF for largest $v$ = 18,546; $P < 0.001$
On 2 DF for smallest $v$ = 15,614; $P < 0.001$

*a DF, degrees of freedom.*

prior to treatment can be the basis of determining the most appropriate treatment for individual patients.

$L_I$ or $v$ is easier to obtain than $T_{pot}$ due to the shorter sampling time and less complex analysis. In this data set, $T_{pot}$ was generally a stronger predictor than $v$, implying that the extra information derived from obtaining $T_S$ is important. Median values were generally used to divide data into groups. For $v$, the median value was not predictive. It was apparent that higher values of $v$ were associated with a poor outcome (Fig. 2), and this was verified statistically (Tables 3 and 4).

Therefore, whereas almost any $T_{pot}$ selected for categorical grouping would result in a statistically significant difference in groups (not shown), $v$ was limited to selecting a group of patients that would do poorly.

With the documented heterogeneous nature of osteosarcoma, it is not surprising that heterogeneity of kinetic parameters was encountered, and results are similar to those reported by Rew et al. (9). That other studies report modest heterogeneity is not contradictory (7, 8). The degree of intratumoral heterogeneity may vary based on the histological type, size, or other factors. The possible impact of heterogeneity needs to be determined for each tumor type being evaluated for kinetic parameters or other possible predictors. In tumors believed to have modest heterogeneity, one sample may be quite representative of the entire tumor, while in more heterogeneous tumors, multiple samples may improve the reliability of the assay. Information designating the best location to sample tumors is also important. It is encouraging that in an extremely heterogeneous tumor such as osteosarcoma that kinetic parameters can be meaningful predictive assays.
REFERENCES


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