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Determination of subcutaneous tumor size in athymic (nude) mice*

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Summary. The athymic (nude) mouse is a useful model for studying the biology and response to therapies of human tumors in vivo. A survey of recent literature revealed the use of 19 different formulas for determining the size of subcutaneous tumors grown as xenografts in nude mice (2 for determining tumor area, 3 for tumor diameter, and 14 for calculating tumor volume). We compared the volumes, areas, and diameters predicted by each of the 19 formulas with the actual weights of 50 tumors ranging from 0.46 to 22.0 g established in nude mice as xenografts from human cell lines. In addition to determining how well each formula predicted relative tumor size, we analyzed how well each formula estimated actual tumor mass. The ellipsoid volume formulas ($\pi/6 \times L \times W \times H$ and $\frac{1}{2} \times L \times W \times H$) were best for estimating tumor mass (r = 0.93), whereas measurements of diameter correlated poorly with tumor mass (r <0.66). Although determination of tumor area correlated well with tumor mass in small tumors (r = 0.89), correlations of area with tumor mass for large tumors were poor (r = 0.41). We conclude that determination of the ellipsoid volume from measurements of three axes consistently yields the most accurate estimations of both relative and actual tumor mass.

Introduction

The ability of human tumors to grow as xenografts in athymic (nude) mice provides a useful model for their study. Measurements of tumor size over time can be used to construct growth curves, which yield unique data on the biology of human tumors in an in vivo environment [23, 42, 53, 59, 65, 67]. In addition, the natural growth pattern of a particular neoplasm can be used as a standard against which the effects of chemotherapeutic agents [3, 8, 12, 18, 19, 25, 44–46, 64], hormones [11, 43, 62], growth factors [55], biological response modifiers [5, 10, 33, 66], monoclonal antibodies [16, 17, 38, 61], or radiation [36, 39, 60] can be measured. To insure accuracy in constructing growth curves, it is important to use a reliable method for determining tumor size.

A survey of recent literature revealed that investigators have used a wide variety of methods to determine the size of tumor xenografts (Table 1). Believing change in tumor diameter to be representative of overall tumor growth, some authors measure only one dimension of the tumor or use the average of two dimensions. Others calculate tumor area from measurements of two perpendicular diameters and assume that the area is proportional to the volume. Still others use one of a variety of formulas that calculate tumor volume from either two or three dimensional measurements of the tumor.

Clearly, the use of a method for determining tumor size that best correlates with actual tumor mass would be optimal. However, although several studies have evaluated different methods for determining tumor size, none have compared all of them (Table 2). In this study we evaluated the 19 different formulas found in the literature to determine those that best estimated relative tumor size and those that most accurately calculated actual tumor mass.

Materials and methods

Growth of cells in nude mice. Human neuroblastoma or primitive neuroectodermal tumor (PNET) cell lines were established in our laboratory or obtained from the originator of the cell line. Tumors used in this study were derived from 21 different neuroblastoma and PNET cell lines [50]. Cell lines were maintained in vitro at 37° C in a humidified 5% CO_2 atmosphere in RPMI 1640 with 10% fetal calf serum (FCS). Cells were tested and found to be free of mycoplasma by culture and by DNA staining [41]. For injection into nude mice, cultured cells were removed from tissue culture flasks using Pucks saline A with 1 mM EDTA and 10 mM HEPES [49] and were washed and resuspended in Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+} (PBS). Cell clumps were dissociated by gentle pipetting with a Pasteur pipette. Cell concentration and viability were determined using 0.06% trypan blue, and 10^8 cells were injected subcutaneously through 22-gauge needles between the scapulae of 6- to 12-week-old BALB/c nu/nu mice (NCI Frederick Cancer Research Facility, Frederick, Md). Mice were housed in a laminar flow caging system (Thoren Caging Systems, Inc., Hazleton, PA), and all food, bedding, and water were autoclaved.

Determination of tumor size. A total of 50 tumors were measured just prior to the sacrifice of the mice. Tumors that grew in irregular shapes were visually divided into two, three, or four lobes of similar dimension; these lobes

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Table 1. Formulas for calculating tumor size

Formula[a]	Papers (*n*)	Total (%)	r^b	Mean square error[c]	References
Volume of a rectangular solid:					
L × W × H	15	7.0%	0.93	121.9	6, 30, 38
$L \times W^2$	2	0.9%	0.82	484.2	66
Ellipsoid volume, assuming $\pi = 3$:					
$1/2 \times L \times W^2$	48	22.5%	0.82	43.0	43, 46, 65
1/2 × L × W × H	7	3.3%	0.93	5.1	8, 9, 47
Ellipsoid volume:					
$\pi/6 \times L \times W \times H$	13	6.1%	0.93	5.2	2, 10, 51
$\pi/6 \times [(L + W)/2]^3$	6	2.8%	0.85	79.8	25, 35
$\pi/6 \times L \times W^2$	5	2.4%	0.82	52.0	14, 15, 58
$\pi/6 \times (L \times W)^{3/2}$	1	0.5%	0.85	71.8	54
$0.4 \times L \times W^2$	11	5.2%	0.82	18.4	1, 36, 67
Spheroid volume:					
$4/3\pi \times r^3$	4	1.9%			20, 55, 56
r = L/2			0.77	291.7	
r = (L + W)/2			0.85	79.8	32, 33
Miscellaneous volumes:					
$4/3\pi \times L \times W^2 \times 1/10$	2	0.9%	0.82	21.4	12
1/3 × L × W × H	1	0.5%	0.93	20.3	34
$1/2\pi \times L \times W \times H$	2	0.9%	0.93	552.7	16, 57
Area:					
L × W	20	8.9%	0.88	6.7	5, 62, 63
$\pi/4 \times L \times W$	1	0.5%	0.88	11.4	26
Diameter:					
L	41	19.3%	0.63	53.0	31, 52, 53
(L + W)/2	22	10.3%	0.65	59.4	24, 40
$(L \times W \times H)^{1/3}$	3	1.4%	0.66	68.0	4, 22, 28
No Formula Stated	10	4.7%			13, 39, 42

[a] In the literature surveyed, different notations were frequently used to convey these formulas. To simplify the table, we have represented all mathematically equivalent formulas with a single standardized notation
[b] Pearson's correlation coefficient
[c] Formula of mean square error $= 1/(n-1) \times \Sigma(\text{actual mass} - \text{calculated mass})^2$

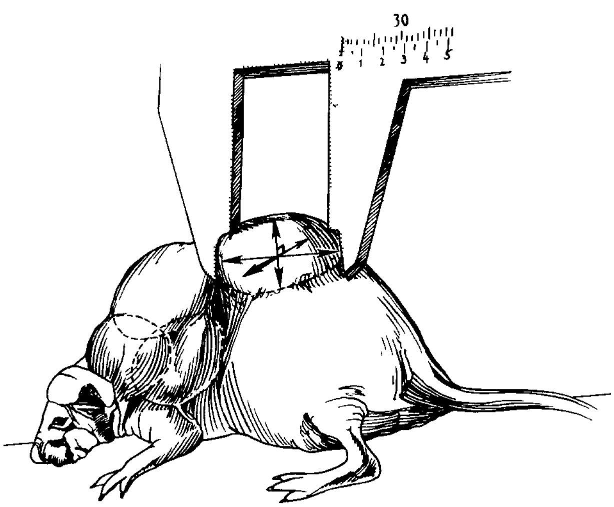

Fig. 1. Illustration of the method used to measure subcutaneous tumors. Multi-lobed tumors were visually separated into regions of similar dimension, and the length (longest dimension), width (shorter dimension, parallel to the mouse body), and height (diameter of tumor perpendicular to the length and width) of each lobe were measured with calipers

were roughly block-like in shape. The length (the longest dimension), width (the distance perpendicular to and in the same plane as the length), and height (the distance between the exterior tumor edge and the mouse's body) of each tumor or lobe was measured with vernier calipers (Fig. 1). Mice were sacrificed by cervical dislocation; the tumor was then dissected free of the skin and body tissue and weighed on a digital balance. Tumors that did not grow entirely subcutaneously (i.e., those that invaded into deeper tissues) were excluded from this study. The median tumor mass was 9.35 g (range, 0.46–22.0 g).

Calculation of tumor size. The diameters, areas, and volumes of all tumors were calculated using each of the formulas in Table 1. In multi-lobed tumors, the diameters, areas, and volumes of the different lobes were calculated separately, and the final value was determined by summation of all values for each lobe. In addition, three tumors of the same cell line were measured by this method at biweekly intervals from the time of injection.

The volumes of representative neuroblastoma and PNET xenografts were determined by water displacement, and their masses were measured on a digital balance. In concordance with previous observations of different tumors [21, 37], the mass and volume were found to be identical (i.e., the density of these tumors was 1 g/ml).

Table 2. Previous studies comparing methods of measuring tumors in mice

Formulas studied	Correlation with mass or volume	Animal model	Additional information	Conclusions	References
$V = L \times W^2$	$r = 0.93$	nude mouse	tumors were spherical	correlation weakened for tumors >20 g	48
Maximal diameter Width Perimeter	N/A N/A N/A	mouse	X-rays measured tumors were spherical	all methods correlated with each other, but diameter was preferred	27
$V = 0.4\,(L \times W^2)$	data not shown	mouse	tumors were spherical	diameter was inaccurate a correction factor (0.4) was needed to estimate actual mass	1
$V = L \times W^2/2$ $V = \pi/6\,(L \times W \times H)$ $V = 4/3\pi\,(L \times W^2)/10$	$r = 0.98-0.99$ $r = 0.98-0.99$ $r = 0.98-0.99$	nude mouse		all correlated well, but $L \times W^2$ was preferred	12
$V = \pi/6\,(L \times W \times H)$ $V = L \times W^2/2$ $A = L \times W$ Diameter H_2O displacement	$r = 0.97-0.99$ $r = 0.97-0.99$ $r = 0.97-0.99$ $r = 0.72-0.77$ $r = 1.0$	nude mouse		volume formulas estimate actual displacement volumes better than area formulas	21
$V = \pi/6 \times L \times W \times H$ $A = L \times W$	N/A N/A	nude mouse	the 3 tumor dimensions were proportional throughout growth L approximately = H	area and volume are proportional	59
$V = (L \times W^2)/2$	mass and volume were within 3% of each other	nude mouse			37
$V = 1/3\,L \times W \times H$	data not shown	nude mouse	formula derived by correlating actual mass with several formulas		34
$A = L \times W$	$r = 0.62$ $r = 0.72$	nude mouse	2 cell lines correlated separately	area found to be good estimate of tumor size	29
$\pi/6 \times L \times W \times H$	$r = 0.99$	nude mouse	tumors were between 0.05 and 6 g	this formula estimated relative and actual mass well	17

Comparing estimations of relative and actual tumor size. Formulas were compared for their ability to estimate both relative tumor size and actual tumor mass accurately. To determine the accuracy of each formula in calculating *relative* tumor size, we determined the Pearson correlation coefficient for the calculated tumor size derived from each formula compared with the actual mass of the tumor [7]. To determine accuracy in predicting *actual* tumor mass (or volume), we calculated the mean square error of the difference between actual and calculated mass using the formula for variance:

$$1/(n\text{-}1) \times \Sigma\ (\text{actual mass} - \text{calculated mass})^2$$

where n = the total number of tumors [7].

Results

We conducted a survey of over 200 journal articles published between 1966 and 1988 on the growth of tumors in mice. Although most of the papers involved subcutaneous nude mouse xenografts, a few studied spontaneous tumors in mice. We found 3 methods for determining tumor diameter, 2 methods for deriving tumor area, and 14 formulas for calculating tumor volume (Table 1). The distribution of formula use (as represented by the literature surveyed) is shown in Fig. 2: 55% of the investigators monitored tumor growth using tumor volume calculations, 31% measured tumor diameter, 9% measured area, and 5% published tumor volume growth curves but did not describe how the volume was derived.

We compared all of the formulas found in the literature for their ability to predict the *actual* tumor mass and the *relative* tumor size for 50 tumors grown subcutaneously in nude mice. All methods for calculating tumor diameter were poor at estimating both relative size and actual tumor

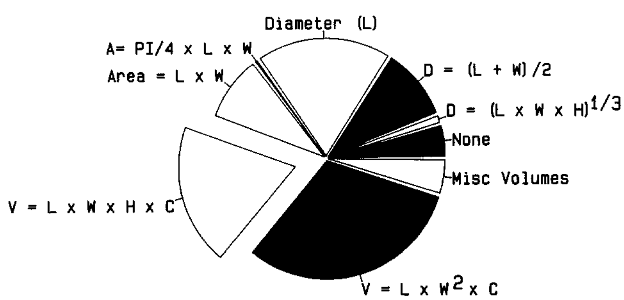

Fig. 2. Distribution of the types of formulas used in 214 articles that measured and monitored tumor growth in mice. Diameter formulas were used in 31% of the papers, area formulas in 9%, and in 55% of the papers tumor volume was measured using one of several formulas. Miscellaneous volume formulas were: $\pi/6[(L + W)/2]^3$ (2.9%), $\pi/6(L \times W)^{3/2}$ (0.5%), and $4/3\,\pi \times r^3$ (1.9%). In the volume formulas ($L \times W \times H \times C$) and ($L \times W^2 \times C$), C represents a constant such as ½, ⅓, 0.4, $\pi/6$, or $4/3\,\pi\,(1/10)$. A = area, D = diameter

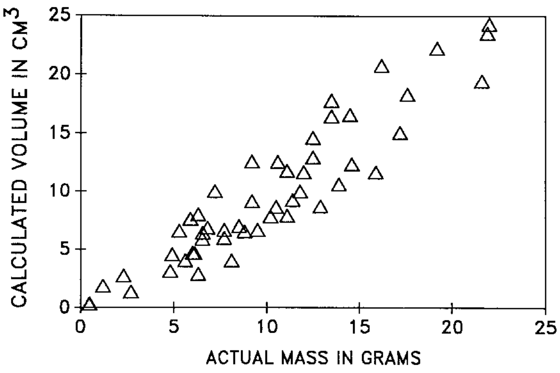

Fig. 3. Correlation of the tumor volume calculated by the formula π/6(L × W × H) with the actual tumor mass ($r = 0.93$)

mass (Table 1). Of all diameter formulas, the geometric mean $(L \times W \times H)^{1/3}$ predicted sizes that correlated best with actual tumor mass ($r = 0.66$).

The Pearson correlation coefficients revealed no large disparities in the accuracy with which different tumor volume formulas predicted relative tumor sizes (Table 1). Formulas that differed from L × W × H by only 1 constant correlated best with tumor mass ($r = 0.93$), whereas those based on the equation $L \times W^2$ showed the poorest correlation of the volume formulas ($r = 0.82$). Comparison of the mean square errors between the actual and predicted tumor masses (Table 1) showed that the formulas that best estimated actual tumor mass were:

½ × L × W × H and π/6 × L × W × H.

Figure 3 shows the good correlation between tumor volumes calculated by the formula π/6(L × W × H) and the actual tumor masses.

To determine if certain formulas are better at estimating the relative size and actual masses of small vs large tumors, we calculated Pearson's correlation coefficients as well as variances between actual and predicted masses for the ten tumors weighing, <6 g and the ten weighing >17 g. Both formulas used to calculate tumor area estimated relative tumor size and actual tumor mass quite well when the entire population of 50 tumors was considered (Table 1) or when tumors weighing <6 g were considered (Table 3). However, for tumors weighing >17 g, area correlated poorly with actual tumor mass ($r = 0.41$), and the mean square errors between the actual masses and estimated areas were quite large (Table 3). By contrast, tumor volume formulas were much more accurate in predicting

Table 3. Select volume and area calculations comparing large and small tumors

Formula	Tumors <6.0 g		Tumors >17.0 g	
	r^a	Mean square error[b]	r^a	Mean square error[b]
Volume:				
1/2 × L × W × H	0.88	1.3	0.79	37.9
π/6 × L × W × H	0.88	1.4	0.79	36.0
Area:				
L × W	0.89	3.9	0.41	40.4
π/4 × L × W	0.89	1.3	0.41	81.2
Diameter:				
L	0.69	2.8	0.16	249.8
(L + W)/2	0.73	3.4	0.20	263.7
$(L \times W \times H)^{1/3}$	0.73	1.3	0.20	46.4

[a] Pearson's correlation coefficient
[b] Formula of mean square error = $1/(n-1) \times \Sigma(\text{actual mass} - \text{calculated mass})^2$

Table 4. Comparison of multi- and single-lobed tumors using selected formulas

Formula	Multi-lobed tumors		Single-lobed tumors	
	r^a	Mean square error[b]	r^a	Mean square error[b]
Volume:				
1/2 × L × W × H	0.92	7.32	0.93	8.50
π/6 × L × W × H	0.92	7.25	0.93	8.99
Area:				
L × W	0.90	6.93	0.84	9.09
π/4 × L × W	0.90	11.72	0.84	7.14
Diameter:				
L	0.77	63.90	0.72	3.49
(L + W)/2	0.79	72.21	0.82	3.03
$(L \times W \times H)^{1/3}$	0.77	84.77	0.90	2.49

[a] Pearson's correlation coefficient
[b] Formula of mean square error = $1/(n-1) \times \Sigma(\text{actual mass} - \text{calculated mass})^2$

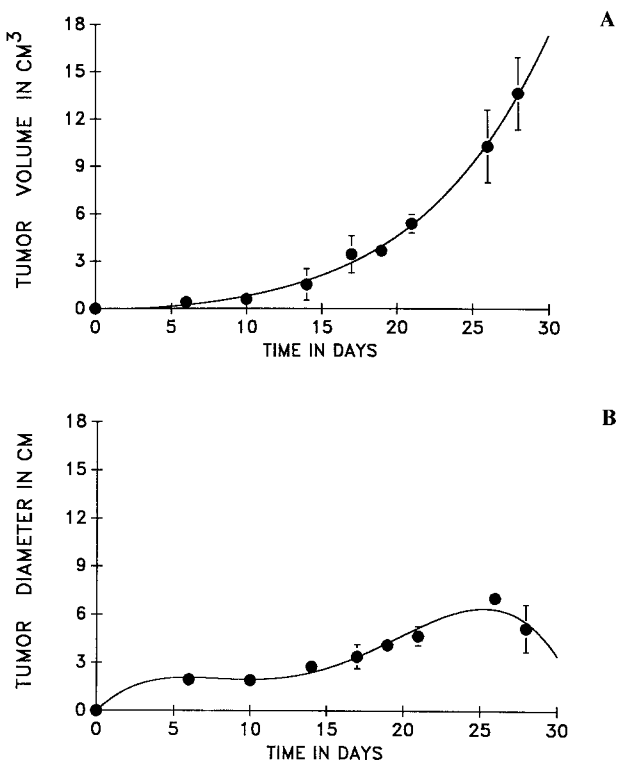

Fig. 4. Tumor growth curves constructed using two different methods for measuring tumor size. Three nude mice were injected s.c. with the SK-N-AS neuroblastoma cell line, and their xenografts were measured at the same points in time. **A** shows a growth curve using tumor size calculated by the volume formula π/6(L × W × H); **B** shows a growth curve using tumor diameter

relative and actual tumor size for tumors weighing > 17 g (Table 3).

We also wished to assess whether the visual division of multi-lobed tumors into separate lobes for measurement accurately determined the relative sizes and actual mass of the tumors. We compared correlation coefficients and mean square errors for the 19 multi-lobed tumors to the same parameters determined for the 31 single-lobed tumors using representative volume, area, and diameter formulas. We found no significant difference in the values, except that the mean square errors between actual mass and calculated diameter were larger for multi-lobed than for single-lobed tumors (Table 4).

We compared the effect of using formulas with poor or good correlations to tumor mass in the construction of longitudinal growth curves. Tumor size calculated by the volume formula $\pi/6(L \times W \times H)$ or by the measurement of simple diameter was plotted vs time for three nude mouse xenografts. As shown in Fig. 4, the use of a formula that correlates poorly with tumor mass (such as diameter) can result in a growth curve vastly different from that obtained with a formula that correlates well with tumor mass.

Discussion

A large number of different methods are presently used for documenting changes in nude mouse tumor size. Most investigators measure (usually with calipers) the dimensions of a palpable subcutaneous tumor. One of the 19 formulas shown in Table 1 is then used to calculate the diameter, area, or volume of the mass. Unfortunately, the lack of standardization of the formulas used in the current literature seems only to propagate the use of still more formulas. Several studies have evaluated different methods of mouse tumor measurement, and we have reviewed these in Table 2.

In this study we used the measurements and actual masses of 50 tumors grown subcutaneously in nude mice to evaluate each of the 19 formulas found in Table 1. By correlating the calculated diameter, area, or volume with the actual mass (which equals the water displacement volume), we determined which methods best estimate relative tumor size. By finding the variance between the actual mass and the calculated volume, area, or diameter, we found which formulas best predict the actual tumor mass. Because we used tumors of various shapes and sizes, we believe that our conclusions have widespread applicability.

Although Pearson correlation coefficients between calculated tumor size and actual mass did not reveal that any one particular formula was distinctly superior for estimating relative tumor size, they did identify formulas that were clearly inferior in predicting relative tumor size. The second most frequently used formula in our survey of the literature, simple diameter (L), proved to be the single worst method for determining relative tumor size (Table 1), and it produced growth curves that were inaccurate representations of actual tumor growth (Fig. 4). Our data suggest that the variability of tumor shapes precludes using the measurement of a single dimension for the accurate determination of tumor size. For example, if the diameters of a spherical tumor are compared with those of a long, thin, flat tumor, the first underestimates tumor size relative to the second.

Tumor area formulas predicted both relative tumor size and actual tumor mass well when the entire population of 50 tumors was considered (Table 1). Area estimations should be used with caution, however, since they are excellent at estimating the relative size and actual mass of small tumors but do not accurately estimate either relative size or actual mass for large tumors (Table 3).

Most calculations of tumor volume were reasonable estimates of relative tumor size, but the formula $L \times W \times H \times C$, where C is a constant, stood out as the best (Table 1). For the latter formula, the correlations between actual and predicted mass weakened when only tumors larger than 17 g were considered, but the *r* values did not decrease as much as they did for area formulas (Table 3). Although many volume formulas predicted relative tumor size well, only a few accurately estimated actual tumor mass. When $C = \pi/6$ or $1/2$, the formula $L \times W \times H \times C$ best predicted actual tumor mass; as $\pi/6 = 1/2$ (assuming $\pi = 3$), the two formulas are interchangeable for practical purposes.

Our results show that calculation of tumor volume using three-dimensional measurements of a tumor yields the most accurate estimation of actual and relative size for tumors grown subcutaneously in nude mice. We also showed that the separate measurement of each lobe in multi-lobed tumors enables the accurate determination of total tumor volume. Of the 19 formulas tested, $L \times W \times H \times C$, where $C = \pi/6$ or $1/2$, best represented the actual mass (and volume) of a wide spectrum of tumor shapes and sizes. Other investigators [12, 17, 21, 59] have also found that this formula, or its equivalent, produces accurate results. The use of this formula for future studies of tumor size in nude mice would insure the accuracy of such studies and enhance the comparability of data between laboratories.

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