The Antineoplastic Effect of Vitamin D in Transgenic Mice With Retinoblastoma

Daniel M. Albert, Dennis M. Marcus, Jeffrey P. Gallo, and Joan M. O'Brien

Vitamin D has been shown to inhibit growth of human retinoblastoma in tissue culture and nude mouse heterografts. We have described a heritable transgenic mouse model of retinoblastoma. Forty-six 8–10-week-old transgene-bearing mice were injected intraperitoneally for 5 wk. Experimental animals received 0.05 μg (15 animals) or 0.025 μg (15 animals) of vitamin D. Sixteen control animals received only a mineral oil vehicle. Eyes were enucleated at 5 mo and were examined histologically by two investigators in a masked fashion. All control animals demonstrated bilateral involvement of retinoblastoma. Four eyes in the low-dose group and six eyes in the high-dose group had no evidence of retinoblastoma. Eyes treated with vitamin D showed less extensive involvement of the retina by retinoblastoma. Vitamin D-treated animals demonstrated tumors confined to the retina, whereas control animals demonstrated larger tumors, more often invading the vitreous, anterior chamber, and choroid. Thus, Vitamin D inhibited the growth and local extension in a dose-dependent fashion. Invest Ophthalmol Vis Sci 33:2354–2364, 1992

Retinoblastoma, the most common primary ocular malignancy of childhood, is a prototypical heritable cancer. Treatment of this cancer with external beam radiation, plaque radiation, cryotherapy, photocoagulation, or enucleation are locally effective therapies. These therapies are well accepted and successful but are destructive and may often compromise visual function. In contrast, the role of chemotherapy in the treatment of human retinoblastoma is controversial. Although adjuvant chemotherapy and external beam radiation have been used successfully in patients with metastatic disease, the efficacy of adjuvant therapies for metastatic and nonmetastatic retinoblastoma is not well defined. Although encouraging results from experimental and clinical studies exist, additional evaluation of various chemotherapeutic drugs is needed.

We recently described the first heritable model of retinoblastoma in transgenic mice. The retinal expression of a viral oncogene, simian virus 40 T antigen, leads to the development of bilateral ocular tumors with features identical to those of human retinoblastoma. This murine model provides a unique opportunity to evaluate the antineoplastic effects of various agents.

Vitamin D is one of the most important biologic regulators of calcium and phosphorous metabolism. Vitamin D's mechanism of action is mediated through receptors that are present in a variety of tissues. Receptors for vitamin D also are present in tissues not involved in systemic calcium/phosphorous metabolism, such as the retina. In addition to its role in mineral homeostasis, the active form of vitamin D, 1,25-dihydroxycholecalciferol (vitamin D3), has been shown to influence the proliferation and differentiation of several tissues.

The role of vitamin D as an antineoplastic agent has been demonstrated by studies that show several human cancer cell lines are inhibited by vitamin D and its metabolites. Furthermore, vitamin D has been shown to suppress the growth of human solid xenograft tumors in vivo. The antineoplastic action of this hormone is mediated by its receptor, which affects gene expression in a manner analogous to other steroid hormones.
Table 1. Dose limiting toxicity

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Mean pretreatment weights</th>
<th>Mean posttreatment weights</th>
<th>Percent change</th>
<th>Mean pretreatment calcium</th>
<th>Mean posttreatment calcium</th>
<th>Percent change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.8 g</td>
<td>28.9 g</td>
<td>+22%</td>
<td>9.25 mg/dl</td>
<td>9.55 mg/dl</td>
<td>+3%</td>
</tr>
<tr>
<td>0.05 µg</td>
<td>20.3 g</td>
<td>17.1 g</td>
<td>-16%</td>
<td>9.48 mg/dl</td>
<td>14.03 mg/dl</td>
<td>+48%</td>
</tr>
<tr>
<td>0.1 µg</td>
<td>21.9 g</td>
<td>16.6 g</td>
<td>-24%</td>
<td>9.35 mg/dl</td>
<td>16.03 mg/dl</td>
<td>+58%</td>
</tr>
<tr>
<td>0.2 µg</td>
<td>19.2 g</td>
<td>15.4 g</td>
<td>-20%</td>
<td>9.35 mg/dl</td>
<td>15.35 mg/dl</td>
<td>+58%</td>
</tr>
</tbody>
</table>

n, number of animals in each group, excluding those that did not complete the study.

D2 and D3. Human retinoblastoma Y-79 cells possessed vitamin D receptors and demonstrated growth inhibition with this hormone. In addition, vitamin D2 and D3 decreased tumor growth of subcutaneous human retinoblastomas in athymic mice. In the present study, we administered vitamin D3 to transgenic mice with retinoblastoma to evaluate its antineoplastic effects in a more relevant animal model.

Materials and Methods

Animals

These procedures in this study were consistent with the ARVO Resolution on the use of Animals in Research. Animals were confirmed to be transgene bearing by DNA tail blot analysis for SV40 T-antigen DNA prior to inclusion in the study, as previously described. Experimental mice ranged in age from 8-10 wk and weighed between 17 and 30 g. At this age, we can first demonstrate histologically localized foci of tumor that are limited to the inner nuclear layer. All mice were maintained on a low calcium diet (0.1% Calcium Purified; Purina Mills, St. Louis, MO) to minimize the toxicity of hypercalcemia.

Calcitriol Treatment

Pure crystalline vitamin D3 (1,25-dihydroxycholecalciferol; provided by Dr. Milan Uskokovic, Hoffman LaRoche, Nutley, NJ) was dissolved in 100% ethanol and stored in amber bottles under argon gas at -70°C. Stock solutions were prepared at the onset of the study and then every 10 days because of the short shelf life of vitamin D. Prior to injection, vitamin D was diluted in a mineral oil vehicle. Vitamin D or a mineral oil vehicle was injected intraperitoneally with a 25 G needle. Animals were injected 5 d a week for 5 wk.

Dose Limiting Toxicity

To assess the toxicity of vitamin D3, 30 transgenic mice were administered 0.5 ml of a mineral oil vehicle (four animals), or 0.05 µg (eight animals), 0.1 µg (10 animals), and 0.2 µg (eight animals) of vitamin D3 diluted in 0.5 ml of a mineral oil vehicle. Toxicity was assessed by survival, daily weights, and serum calcium levels obtained from selected control and treatment group mice prior to the first injection and at the end of the fifth week of treatment. Blood samples were collected from the tails of mice with microliter capillary tubes and were centrifuged to separate the serum.

Tumor Inhibition

Based on the results obtained from the above evaluation of vitamin D toxicity, 46 transgenic mice were administered vitamin D or a mineral oil vehicle. Sixteen animals received 0.5 ml of a mineral oil vehicle, 15 animals received doses of 0.025 µg of vitamin D in 0.5 ml of mineral oil, and the remaining 15 mice received doses of 0.05 µg of vitamin D in 0.5 ml of mineral oil. Toxicity was assessed by survival, records of daily weights, and serum calcium levels. Two animals from each study group were selected for serum calcium levels prior to the first injection, at the end of the study and then every 10 days because of the short shelf life of vitamin D. Prior to injection, vitamin D was diluted in a mineral oil vehicle. Vitamin D or a mineral oil vehicle was injected intraperitoneally with a 25 G needle. Animals were injected 5 d a week for 5 wk.

Table 2. Vitamin D toxicity

<table>
<thead>
<tr>
<th>Vitamin group</th>
<th>Mean pretreatment weights</th>
<th>Mean posttreatment weights</th>
<th>Percent changes</th>
<th>Mean pretreatment calcium</th>
<th>Mean posttreatment calcium</th>
<th>Percent changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.6 g</td>
<td>24.6 g</td>
<td>+26%</td>
<td>9.0 mg/dl</td>
<td>9.2 mg/dl</td>
<td>+2%</td>
</tr>
<tr>
<td>0.025 µg</td>
<td>21.3 g</td>
<td>23.4 g</td>
<td>+10%</td>
<td>8.9 mg/dl</td>
<td>13.0 mg/dl</td>
<td>+46%</td>
</tr>
<tr>
<td>0.05 µg</td>
<td>20.0 g</td>
<td>19.6 g</td>
<td>-2%</td>
<td>8.9 mg/dl</td>
<td>13.1 mg/dl</td>
<td>+47%</td>
</tr>
</tbody>
</table>

n, number of animals in each group, excluding those that did not complete the study.
the third experimental week, and at the end of the fifth week. All animals were killed at 5 mo of age (an age at which transgenic mice demonstrate confluent or extensive tumor involvement).

All eyes were enucleated and fixed in 10% formalin for histopathologic examination. Tissue sections from three separate levels within the eye, including the optic nerve, were stained with hematoxylin-eosin. Slides were coded and investigators remained masked with regard to treatment.

Three slides, one from each level within the eye, were evaluated independently by two of the authors (DA and JG) with respect to the following parameters.

- Degree of retinal involvement as determined by the presence of a single focus, multiple foci, or confluent involvement. Involvement was considered confluent only if there was no identifiable retina on histologic examination. Retinal involvement also was estimated by the percent of uninvolved retina, retina with tumor limited to the inner nuclear layer, and retina totally replaced by tumor (full thickness). The largest cross sectional area of tumor involvement, estimated microscopically with a 100 point reticle, provided additional information.

- Involvement of ocular structures, including the vitreous, lens, ciliary body, iris, angle, anterior chamber, cornea, choroid, retinal pigment epithelium, optic nerve, and extraocular extension.

- Tumor morphology determined by percent of tumor calcification, percent of tumor necrosis, proportion of undifferentiated tumor, Homer Wright

### Table 3. Retinal involvement

<table>
<thead>
<tr>
<th>Tumor foci</th>
<th>Mean largest x-sectional area</th>
<th>Extent of retinal involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absent</td>
<td>Single</td>
</tr>
<tr>
<td>Control</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>0.025 µg</td>
<td>27</td>
<td>4</td>
</tr>
<tr>
<td>0.05 µg</td>
<td>26</td>
<td>6</td>
</tr>
</tbody>
</table>

\( n \), number of eyes examined in each study group.

*Statistically significant \((P < 0.040)\), by nonparametric, ranked data.

† Statistically significant \((P < 0.011)\), by nonparametric, ranked data.

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Fig. 2. (A) Control transgenic mouse eye demonstrating retinoblastoma filling globe. (C, cornea; hematoxylin-eosin, original magnification X7.88.) (B) Note prominence of vascularization. (H-E, original magnification X31.5).

rosettes, Flexner-Wintersteiner rosettes, number of mitoses per six high power fields, and degree and character of inflammation of the retina and ocular structures.

Statistics

The mean of the largest cross-sectional areas per eye, mitoses per six high power fields, and tumor mor-
The proportion of each group displaying involvement of the vitreous, ciliary body, iris, angle, anterior chamber, and cornea, as well as tumor calcification and extraocular extension, were evaluated with the chi-squared test. The Fisher exact test was used to interpret the significance of lens involvement and the proportion of eyes in each group with identifiable tumor because expected values in the experimental groups were too small for chi-squared analysis.

 Ranked data, including tumor foci, estimations of percent tumor necrosis, and the extent of optic nerve and choroidal invasion, were evaluated for statistical significance by the Kruskal-Wallis ANOVA by rank. In the presence of significant main effects, pairwise differences were evaluated by Bonferroni-adjusted Mann-Whitney U tests.

 Bonferroni adjustments were applied to the Mann-Whitney U, chi-squared, and Fisher exact tests to compensate for additive type I error resulting from multiple comparisons.

Results

Dose Limiting Toxicity

The toxicity of vitamin D was manifested by hypercalcemia, weight loss, and death. All control animals that received mineral oil vehicle survived five weeks of treatment. Mortality rates for the 0.05 μg, 0.1 μg, and 0.2 μg groups were 25%, 50%, and 50% respectively. The serum calcium levels of mice tested prior to treatment ranged from 8.9-9.7 mg/dl (n = 12). Control group serum calcium levels after 5 wk of treatment ranged from 9.2-9.9 mg/dl (n = 3), while mice that received 0.05 μg of vitamin D had calcium levels in the range of 13.9-14.2 mg/dl (n = 3). This represents a 48% increase in calcium levels in the 0.05 μg dose group over pretreatment values, and a 47% increase over control post-treatment values. All mice that received vitamin D lost weight progressively during the treatment period. Control mice gained 22% of their average pretreatment weight, while the vitamin D groups lost 16% (0.05 μg), 24% (0.1 μg), or 20% (0.2 μg) of their average pretreatment weight (Table 1). Animals that did not survive the 5 wk study period were not included in the final analysis of weight.

Tumor Inhibition

Two animals in the high-dose group (0.05 μg) and one in the low-dose group (0.025 μg) died prior to the termination of the study, and the eyes were excluded from examination. In addition to the deaths of the three animals, the toxicity of vitamin D again was manifested by weight loss and hypercalcemia. Surviving control and low-dose (0.025 μg) animals gained weight over pretreatment values, 26% and 10%, respectively. High-dose (0.05 μg) animals, completing five study weeks, lost 2% of their average pretreatment weight. All study animals tested had pretreatment calcium levels ranging from 8.5-9.3 mg/dl. Control group serum calcium levels ranged from 8.6-9.6 mg/dl during the 5 wk study period, while animals that received vitamin D had calcium levels in the range of 11.3-14.4 mg/dl (Table 2).

As a result of self-mutilation, one eye in the low dose group and two eyes in the control group were unable to be evaluated. One hundred percent of control animals demonstrated bilateral involvement of the retina by tumor, results consistent with the natural progression of transgenic retinoblastoma. No tumor was evident in four of the low-dose-group eyes (two animals) and six of the high-dose-group eyes (three animals; P < 0.014; Fig. 1).

Degree of Retinal Involvement

Eyes treated with vitamin D showed less extensive involvement of the retina by tumor. Twenty-three percent of eyes in the high-dose group (six; P < 0.011)
Fig. 3 (continued)
Fig. 4. (A) Transgenic mouse eye after treatment with high dose (0.05 μg) of vitamin D$_3$. High dose tumors were more likely to be confined to the inner nuclear layer. (B) Note tumorous foci with intervening area of relatively normal retina. (C) Note absence of blood vessels in retinoblastoma nodules. (Hematoxylin-eosin, original magnifications ×7.88, ×31.5, and ×78.75, respectively.)
Intraocular Extension

Vitamin D appeared to inhibit the local extension of tumor in a dose-dependent fashion. Choroidal involvement was observed in 70% of controls, 33% (P < 0.010) of low-dose eyes, and 31% (P < 0.022) of high-dose eyes. Optic nerve invasion was identified in 25% of controls, 33% of low-dose eyes, and 18% of high-dose eyes. Tumor invaded the vitreous in 90% of control eyes and in 52% (P < 0.007) and 46% (P < 0.002) of low- and high-dose groups, respectively. Ciliary body involvement was identified in 76% of controls, 44% of low-dose groups (P < 0.050), and 38% of high-dose groups. Involvement of angle structures was observed in 50% of controls, 33% of low-dose eyes, and 19% of high-dose eyes. Iris invasion was noted in 77% of controls, 41% of low-dose eyes, and 27% (P < 0.014) of high-dose eyes. The lens was involved in 27% of control eyes and 0% (P < 0.005 in high dose; P < 0.011 in low dose) of treated eyes. Tumor was identified in the anterior chamber of 50% of control eyes, 44% of low-dose eyes, and 31% (P < 0.032) of high-dose eyes. Corneal involvement was observed in 43% of control eyes, 26% of low-dose group eyes, and 12% (P < 0.040) of high-dose group eyes. Extraocular extension occurred in 37% of control group eyes, 30% of low-dose-group eyes, and 23% of high-dose-group eyes (Table 4, Figs. 2-4).

Tumor Morphology

Morphologic examination revealed a higher percentage of undifferentiated tumor in the control group, consistent with the larger size and natural progression of transgenic retinoblastoma. Control tumors were, on average, made up of 47% Homer Wright rosettes and 53% undifferentiated tumor. Low-dose-group tumors were made up of 70% (P < 0.003) Homer Wright rosettes and 30% (P < 0.003) undifferentiated tumor. High-dose-group tumors consisted of 60% Homer-Wright rosettes and 40% undifferentiated tumor. Ninety three percent of the tumors in control group eyes were found to have greater than 5% necrosis, compared to 59% (P < 0.020) of low-dose tumors and 46% (P < 0.005) of high-dose tumors, again in agreement with a higher amount of necrosis found in larger tumors. Tumors were less than 1% calcified in 87% of control group eyes, 74% of low-dose eyes, and 65% of high-dose eyes. The mean number of mitoses per six high power fields was 17.8 for control tumors and 16.2 and 16.0 for low- and high-dose-group tumors, respectively (Table 5, Fig. 2-4).

Discussion

In the present experiments, we have demonstrated that intraperitoneal administration of vitamin D3 inhibits the growth of retinoblastoma in transgenic mice. Vitamin D3 seemed to limit tumor extension. Experimental animals demonstrated tumors confined to the retina, whereas control animals demonstrated larger tumors, more often invading the vitreous, anterior chamber, and choroid. In addition, there was total regression of the tumor in six treated eyes.

By what mechanism does vitamin D3 inhibit retinoblastoma? Many possible mechanisms for the antineoplastic properties of this hormone have been pro-

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Table 4. Intraocular extension

<table>
<thead>
<tr>
<th>n</th>
<th>Choroid</th>
<th>Optic nerve</th>
<th>Vitreous</th>
<th>Ciliary body</th>
<th>Iris</th>
<th>Angle</th>
<th>Lens</th>
<th>Anterior chamber</th>
<th>Cornea</th>
<th>Extraocular extension</th>
</tr>
</thead>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>21 (70%)</td>
<td>4*</td>
<td>27 (90%)</td>
<td>23 (76%)</td>
<td>23 (77%)</td>
<td>15 (50%)</td>
<td>8 (27%)</td>
<td>15 (50%)</td>
<td>13 (43%)</td>
<td>11 (37%)</td>
</tr>
<tr>
<td>0.025 μg</td>
<td>27 (P &lt; 0.01)</td>
<td>9 (25%)</td>
<td>14 (90%)</td>
<td>12 (76%)</td>
<td>11 (77%)</td>
<td>9 (50%)</td>
<td>0 (27%)</td>
<td>12 (50%)</td>
<td>7 (43%)</td>
<td>8 (37%)</td>
</tr>
<tr>
<td>0.05 μg</td>
<td>26 (P &lt; 0.022)</td>
<td>8 (33%)</td>
<td>12 (22%)</td>
<td>10 (52%)</td>
<td>7 (41%)</td>
<td>5 (33%)</td>
<td>0 (44%)</td>
<td>8 (26%)</td>
<td>3 (26%)</td>
<td>6 (30%)</td>
</tr>
</tbody>
</table>

n, number of eyes examined in each study group. * Optic nerve was sectioned in 16 controls, 22 low dose and 17 high dose eyes.
posed. Because of the prominence of calcification in spontaneously regressed tumors, Verhoef suggested that vitamin D may be an effective therapy. The antineoplastic effect of vitamin D is unlikely to be a result of its hypercalcemic actions. High calcium levels seem to stimulate cellular proliferation and dampen the inhibitory properties of vitamin D. This is confirmed by our results that show there were no differences in tumor calcification among control and high- or low-dose transgenic animals. In addition, vitamin D did not appear to cause tumor destruction by necrosis. Immunostimulatory effects of this hormone, in the form of lymphocyte or inflammatory infiltration, were not observed in control or experimental animals.

Vitamin D3 plays an important role in the differentiation of various tissues, such as hematopoietic cells, lymphocytes, and epidermal cells. These differentiating effects have been suggested to account for its antioncogenic properties. Steroid receptor genes seem to control photoreceptor cell differentiation in the developing Drosophila retina, so vitamin D may have inhibited retinoblastoma through its differentiating properties. We did not, however, find vitamin D-treated transgenic tumors to be more differentiated than tumors of comparable size.

The ability of vitamin D to limit expansion of transgenic retinoblastoma may be a result of this hormone’s ability to inhibit angiogenesis. Oikawa and co-workers have shown that the active metabolite of vitamin D3 and a synthetic vitamin D3 analogue inhibits angiogenesis in a dose-dependent manner. Human retinoblastoma grows in uniform collar-like configurations with evidence of necrosis at a constant radius around central blood vessels. Thus, retinoblastoma cells seem to rely on their capacity (or adjacent ischemic retina) to produce angiogenic factors.

Study of the natural progression of transgenic retinoblastoma reveals that mice 3–4 mo of age demonstrate smaller, localized tumors without obvious vascularization or angiogenic activity. Tumors tend to grow at a faster rate after 4 mo of age. At this stage, transgenic retinoblastoma demonstrates less differentiation, more necrosis, and a greater vascular supply. The striking feature of this exponential growth phase appears to be the angiogenic activity in transgenic tumors. Given that the natural history of transgenic retinoblastoma growth seems highly dependent on angiogenesis, vitamin D inhibition of angiogenesis may be an important mechanism in limiting tumor extension.

Vitamin D3 mediates its action via the steroid-receptor complex, which is associated with nuclear DNA, leading to the synthesis or repression of various RNA encoding proteins or transcription factors. Vitamin D3’s interaction with the c-myc promoter leads to a reduction in myc RNA levels. Alteration in the transcription of the myc oncogene causes cell maturation of human leukemic cells in culture. This hormone also has been associated with modulations in c-fos and c-fms oncogene expression. The retinoblastoma susceptibility gene regulates the transcription of oncogenes, such as c-fos, so it is possible that unexplored transcriptional mechanisms also may account for this hormone’s suppression of retinoblastoma.

Our results indicate that vitamin D3 therapy may be helpful in limiting vision-threatening growth and in the prevention and treatment of metastatic human retinoblastoma. Prophylactic chemotherapy combined with locally effective regimens (external beam radiation, plaque radiation, cryotherapy, photocoagulation) may assist in eradicating intraocular tumors and may lower the incidence of metastases. With molecular biologic screening of infants for inheritance of an abnormal retinoblastoma gene, earlier recognition and diagnosis of retinoblastoma will continue. The incidence of metastatic disease has declined and will likely continue to do so, so evaluation of prophylactic therapies are paramount in attempting to eliminate mortality from retinoblastoma.

Because of possible carcinogenic effects of conven-

### Table 5. Tumor morphology

<table>
<thead>
<tr>
<th>n</th>
<th>Undifferentiated tumor</th>
<th>Homer-Wright rosettes</th>
<th>Tumors with &gt;3% necrosis</th>
<th>Tumors with &lt;1% calcification</th>
<th>Mean no. of mitoses per 6 HPF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>30</td>
<td>53%</td>
<td>47%</td>
<td>28 (93%)</td>
<td>26 (87%)</td>
</tr>
<tr>
<td>0.025 μg</td>
<td>23</td>
<td>30% (P &lt; 0.003)</td>
<td>70% (P &lt; 0.003)</td>
<td>13 (59%)</td>
<td>17 (74%)</td>
</tr>
<tr>
<td>0.05 μg</td>
<td>20</td>
<td>40%</td>
<td>60%</td>
<td>9 (P &lt; 0.005)</td>
<td>13 (65%)</td>
</tr>
</tbody>
</table>

n, number of tumors examined in each study group.
tional chemotherapeutic agents, concern has been expressed about the possibility of inducing secondary tumors in this predisposed population.\textsuperscript{146} An obvious advantage of vitamin D therapy is an absence of carcinogenic side effects. Hypercalcemic side effects of this hormone are its main complication in humans and was present in this animal study.\textsuperscript{47} Hypercalcemia may be decreased by calcium-lowering therapies or by other vitamin D analogues with a lessened hypercalcemic effect.\textsuperscript{47} Topical vitamin D therapy probably would eliminate systemic side effects, and this mode of therapy will be investigated in this model. We anticipate that these and other studies with vitamin D will influence the nonsurgical management of human retinoblastoma.

**Key words:** retinoblastoma, transgenic mice, vitamin D, 1,25 dihydroxycholecalciferol, angiogenesis

**References**