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Lifespan extension and cancer prevention in HER-2/neu transgenic mice treated with low intermittent doses of rapamycin

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Target of Rapamycin (TOR) is involved in cellular and organismal aging. Rapamycin extends lifespan and delays cancer in mice. It is important to determine the minimum effective dose and frequency of its administration that still extends lifespan and prevents cancer. Previously we tested 1.5 mg/kg of rapamycin given subcutaneously 6 times per two weeks followed by a two-week break (1.5 × 6/bi-weekly schedule: total of 6 injections during a 4-week period). This intermittent treatment prolonged lifespan and delayed cancer in cancer-prone female FVB/N HER-2/neu mice. Here, the dose was decreased from 1.5 mg/kg to 0.45 mg/kg per injection. This treatment was started at the age of 2 months (group Rap-2), 4 months (Rap-4), and 5 months (Rap-5). Three control groups received the solvent from the same ages. Rapamycin significantly delayed cancer and decreased tumor burden in Rap-2 and Rap-5 groups, increased mean lifespan in Rap-4 and Rap-5 groups, and increased maximal lifespan in Rap-2 and Rap-5 groups. In Rap-4 group, mean lifespan extension was achieved without significant cancer prevention. The complex relationship between life-extension and cancer-prevention depends on both the direct effect of rapamycin on cancer cells and its anti-aging effect on the organism, which in turn prevents cancer indirectly. We conclude that total doses of rapamycin that are an order of magnitude lower than standard total doses can detectably extend life span in cancer-prone mice.

Introduction

Rapamycin is an anti-cancer and anti-aging drug.[1] Rapamycin extends life span in mice.[2-20] Furthermore, rapamycin delays the onset of cancer in mice.[3-7,9-13,15,18] Rapamycin and everolimus (a rapamycin analog) decrease the risk of cancer in humans, who receive these rapalogs to prevent transplant rejection.[1,21-24] Therefore, rapalogs such as rapamycin are considered for both prevention of cancer and extension of healthy life span in humans.[25-31] There is a concern that potential side effects may limit rapamycin use as anti-aging drug. This concern is exaggerated. First, metabolic side effects of high chronic doses seem to be benevolent.[32] In fact, rapamycin extends (not shortens) lifespan in all animal studies. Second, instead of daily treatment, rapamycin can be used intermittently. In organ transplant patients, rapamycin and evirolimus are administrated in high doses daily to achieve full and steady inhibition of mTOR complex 1 (mTORC1). For prevention of aging and its diseases, there may be no need in complete effect. Furthermore, in theory, pulse treatment with rapamycin can improve stem cell function and wound healing.[33] In fact, short-term treatment with rapamycin preserved stem cell function.[2,34-36] Rapamycin can improve and stimulate the immune response[37]

In most studies, mice were treated with 1.5–2 mg/kg rapamycin. Importantly, the clearance of rapamycin is much faster in mice than in humans. For example, in mice levels of rapamycin drop 20-folds the next day after injection,[14] whereas in humans its terminal half-life is about 2.5 d.[38,39] It was estimated that a 1.5 mg/kg injection in mice corresponds to the therapeutic oral dose in humans.[40]

Every other day (e.o.d.) administration of 1.5 mg/kg rapamycin dramatically prevented cancer induced by tobacco-carcinogen.[40] We introduced intervals between treatments: e.o.d. (for practical convenience: 3 times per week) rapamycin was administrated bi-weekly (every other two weeks). Thus, mice were treated with 1.5 mg/kg × 3 times a week for 2 wk followed by a 2-wk break. We showed that this schedule delayed cancer and extended mean and maximal lifespan in mice.[4,6] This treatment was started from a very young age (2 mo). In the current study, treatment was started from the age of 2, 4, and 5 mo. Importantly, the dose of rapamycin was reduced from 1.5 mg/kg to 0.45 mg/kg (**Fig. 1**). We evaluated the effect of low-dose treatment on lifespan and cancer onset.

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Results

Tumor development and lifespan in three control groups

The distribution of survivors was similar in three control groups (**Table 1**). The mean life span, life span of last 10% survivors and maximum life span were similar in all control groups (**Fig. 2A–C**; **Table 2**). Tumor incidence and multiplicity (a number of tumors per mouse), the mean latent period of the first tumor and the incidence of metastases were also similar in all control groups of mice (**Table 3**). There was the tendency to earlier cancer in mice treated with solvent from the earlier age (group C-2) than in C-4 and especially in C-5 groups (**Table 2**) consistent with the observation that injections per se may accelerate carcinogenesis. In C-2 control group, the first tumor was detected by the age of 128 d. By the age of 6 mo mammary carcinomas were detected in 3 of 8 mice in C-2 group (37.5%). In C-4 control group, first tumor was detected at the age of 150 d. At the age of 6 mo tumors were developed in 19 of 32 mice (59.4%). In C-5 control group, by 6 mo of age 9 of 19 mice (47.4%) had tumors. Thus, the rapid development of detectable, macroscopic tumors began by the age of 5–6 mo. Treatment with rapamcyin was started at the age of 2, 4 and 5 mo.

The effect of rapamycin on lifespan

Rapamycin treatment started at the age of 4 and 5 mo significantly increased the mean life span (**Fig. 2B and C**; **Table 2**). The lack of the effect of rapamycin on the mean life span in Rap-2 group can be explained by the death of some mice early in life (**Table 1**; **Fig. 2A**) before cancer developed (cancer-unrelated death), which may be accidental or due to effects of rapamycin at very young age (2 mo). Still in Rap-2 group, rapamycin extended maximal lifespan by 9.6%. Similarly, in Rap-5 group, rapamycin increased the mean life span of the last 10% survivors (**Fig. 2C**; **Table 2**).

The effect of rapamycin on carcinogenesis

In groups Rap-2 and Rap-5, the mean latent period of the first tumor development was significantly increased. The kinetic of tumor incidence in rapamycin-treated mice (R2 and R5) was slower than the kinetic in control animals (**Fig. 2D–F**). Thus, 50% of all control mice developed tumors by the age of 175–180 d (**Fig. 2D and F**), whereas in Rap-2 and Rap-5 groups this period was extended to 200–205 d. These data demonstrate that rapamycin not only increased lifespan, but also reduced mammary carcinogenesis in cancer-prone mice. In Rap-4 group, rapamycin increased the number of mice with multiple metastases (40.6% and 18.8%, correspondingly, $P < 0.02$, exact Fischer test), probably due to an increase in life span of tumor-bearing mice. A number of tumors per animal was similar in rapamycin-treated and control groups (**Table 3**). Yet, 6.3% of C-4 mice were bearing 1–4 tumors per mouse, and 40.6% had 5–6 tumors per mouse, whereas in rapamycin-treated groups these numbers were 15.6% and 24.4%, respectively.

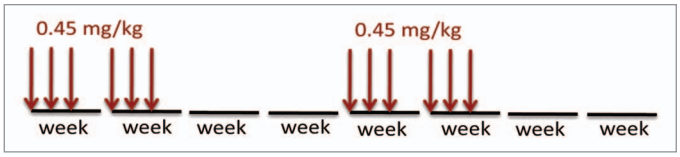

Figure 1. The intermittent low doses treatment. A dose of 0.45 mg/kg rapamycin was injected subcutaneously 3 times per week for 2 wk, followed by a two-week interval.

Discussion

Rapamycin slows down aging, prevents cancer and extends lifespan in mice. Noteworthy, metformin, which affects the AMPK/mTOR pathway, also extends life span in mice and prevents cancer in mice[41-49] Unlike metformin, which is effective mostly when started early in life,[45] rapamycin extends life span when the treatment started both in young and in old animals (**Table 4**). In order to develop low-dose anti-aging therapy for humans, one needs to determine the minimum effective dose of rapamycin. Currently in the clinic, rapamycin is mostly administered daily in high dose. In most animal studies, rapamycin was also used as daily treatment either with food or by injection. Such treatments extended life span (**Table 4**). In some studies, rapamycin was administrated every other day (e.o.d.) and even weekly or biweekly (**Table 4**). Previously, we have tested double intermittent schedule 1.5 mg/kg × 6/4 wk in 2-mo-old inbred[6] and cancer-prone[4] female mice. In both studies, rapamycin delayed cancer, decreased multiplicity of tumors per mice and extended life span. Here the dose was decreased from 1.5 to 0.45 mg/kg. The current study additionally included groups Rap-4 and Rap-5, with treatment started at the age of 4 and 5 mo, respectively. Noteworthy, rapamycin treatment started at the age of 2 mo (but not at 4 or 5 mo) was associated with cancer-unrelated death at a

Table 1. Survival distribution in mice treated with rapamycin and solvent (control) from different age

Group	Age at start, mo	Age of mice, months											
		2	3	4	5	6	7	8	9	10	11	12	13
Control-2	2	12	12	12	11	11	10	9	8	3	1	0	0
Rapamycin-2	2	11	11	10	9	9	8	8	7	5	3	1	0
Control-4	4	-	-	32	32	32	31	29	22	6	2	0	0
Rapamycin-4	4	-	-	32	32	32	32	30	27[a]	13[a]	3	0	0
Control-5	5	-	-	-	19	19	19	19	13	3	1	0	0
Rapamycin-5	5	-	-	-	19	18	18	17	16[a]	12[a]	2	1	0

[a]Significant difference with the corresponding control group: $P < 0.01$ (Fischer exact test)

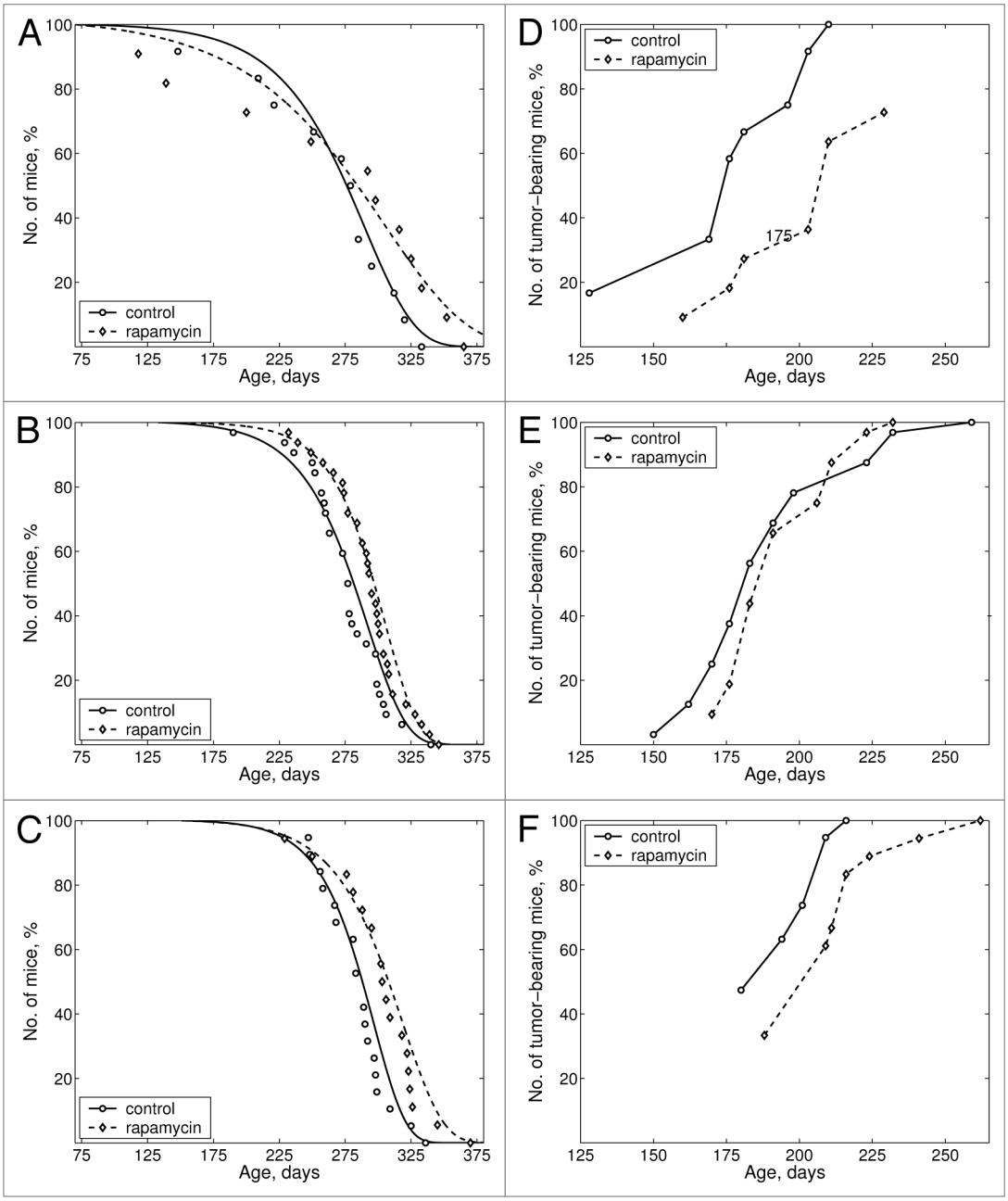

Figure 2. Effects of rapamycin on life span and tumor incidence. (**A–C**) Effects of rapamycin on mice survival. (**D and E**) Effects of rapamycin on tumor yield curves. (**A and D**) Upper panel (groups C-2 and Rap-2): treatment was started at the age of 2 mo. (**B and E**) Middle panel (groups C-4 and Rap-4): treatment was started at the age of 4 mo. (**C and F**) Middle panel (groups C-5 and Rap-5): treatment was started at the age of 5 mo.

Table 2. Parameters of life span in mice treated with rapamycin and solvent (control) from different age

Parameters	Control-2	Rap-2	Control-4	Rap-4	Control-5	Rap-5
No. of mice	12	11	32	32	19	18
Mean life span, d	268 ± 15.2	272 ± 25.5	278 ± 5.4	293 ± 4.7 +5.4%; t = 2.09	285 ± 5.5	304 ± 7.7 +6.7%; t = 2.01
Median life span, d	282	298	278	295	289	305
Mean life span of last 10%, d	333 ± 0	365 ± 0 +9.6%	333 ± 7.3	339 ± 3.8	331 ± 5.5	358 ± 12.5 (+8.2%); t = 1.98
Maximum life span, d	333	365 +9.6%	340	346	336	370 (+10.1%)

The difference with relevant controls is significant, $P < 0.05$.

Table 3. Effects of rapamycin on tumor development

Parameters	Control-2	Rap-2	Control-4	Rap-4	Control-5	Rap-5
No. of mice	12	11	32	32	19	18
1st tumor detection	128	160	150	170	180	188
No. effective mice	12	9	32	32	19	18
No. of tumors	12 (100%)	8 (88.9%)	32 (100%)	32 (100%)	19 (100%)	18 (100%)
Mean latency of the 1st tumor, d	176 ± 7.7	197 ± 8.1 +11.9%	191 ± 4.5	194 ± 3.1	192 ± 3.1	209 ± 4.7[a] +8.9%
Total number of tumors	76	59	212	216	144	123
No. of tumors per mouse	6.3	7.4	6.6	6.8	7.6	6.8
No. of mice with metastases into lung	8 (66.7%)	7 (63.7%)	16 (50.0%)	24 (75.0%)	13 (68.4%)	12 (66.7%)

[a]The difference with relevant controls is significant, $P < 0.05$.

young age, blunting the effect of rapamycin on median (but not maximal) lifespan (**Fig. 1A**). Remarkably, Johnson et al. demonstrated extraordinary life extension by high-dose rapamcyin treatment started at the age 20 d in very short-lived Leigh syndrome mice.[14] So there may be no negative effect in very young age (albeit rapamycin-treated mice growth was slowed down[14]).

In the current study, 0.45 mg/kg rapamycin exerted similar effects as 1.5 mg/kg rapamycin used by us previously, yet, as may be expected, the effects of lower doses were less pronounced.[4] The extension of maximal life span was observed in groups Rap-2 and Rap-5. In groups Rap-4 and Rap-5, rapamycin increased medium lifespan. In group Rap-5, both medium and maximal lifespan were increased. Significant tumor prevention was observed in groups Rap-2 and Rap-5. The extension of the medium lifespan was associated with the extension of lifespan of cancer-bearing mice, explaining an increase of a number of mice with metastasis. In brief, 0.45 mg/kg × 6/biweekly treatment delayed cancer in two groups and extended either maximal or medium lifespan, or both. The difference between groups may depend on potential negative effects of rapamycin injections at very early age (2 mo), direct anti-cancer effect, selection for resistance of premalignant cells, and indirect anti-cancer effect via anti-aging effects of rapamycin. Although many explanations are possible, we cannot provide the evidence. The simplest explanation is that doses used in this study exerted mild effects, which statistical significance varied from group to group. Therefore, in some groups cancer-prevention was not statistically significant, while rapamycin still significantly extended median life span. In general, we can conclude that low-dose treatment exerted modest effects, probably reaching its threshold of statistically significance in small groups of animals.

In this study, we tested the lowest doses/frequencies of rapamycin in mice compared with all previous studies. In heterogeneous mice, rapamycin-containing food extended female medium life span by 13–18%, when treatment started at 600 d[3] and 270 d,[5] respectively. Expressed as life expectancy at 600 d (the age of first exposure to rapamycin), the effect size was 38% for females.[3] In studies that included males and females rapamycin extended life span in females more significantly. One explanation is that the mTOR pathway is less sensitive to rapamycin in males than in females.[50] Given that we treated breast cancer-prone mice, all mice in our study were females.

In study by Johnson,[14] the effect of high-dose daily rapamycin was so dramatic that maximal life span was extended 300% and mean life span 100%.[14] In contrast, e.o.d. rapamycin extended life span just by 38%[14] Yet, it was a special model of mitochondrial disease with extremely short life span in control.[14] To compensate for profound mitochondrial dysfunction, a steady full inhibition of mTOR was necessary. In our studies, cancer was modestly prevented by intermittent low-dose administration of rapamycin, although no comparison with daily doses is available. We assume that higher doses and daily administration would be more potent. Yet, low-dose intermittent rapamycin seems to be a practical approach to prevent cancer and extend life span in healthy human population.

Material and Methods

Animals and experimental design

Homozygous FVB/N HER-2/neu transgenic mice originally obtained from Charles River by the Italian National Research Center for Aging (INRCA) were housed and breed in the Department of Carcinogenesis and Oncogerontology, N.N. Petrov Research Institute of Oncology. Mice received standard laboratory chow and tap water ad libitum.[51] All studies were conducted in accordance with the ethical standards and according to national and international guidelines and have been approved by the authors' institutional review board.

Longevity study

One hundred and twenty-four (124) female FVB/N HER-2/neu mice were under observation. Sixty-one mice received 0.45 mg/kg rapamycin (LC Laboratories) subcutaneously (s.c.) 3 times a week for a period of 2 wk followed by 2-wk intervals starting at the age of 2 mo (11 mice), 4 mo (32 mice) or 5 mo (18 mice). Rapamycin was dissolved in 95% ethanol and then diluted with apyrogenic sterile water to a final concentration of 11.4 μg in 0.1 ml of 2% ethanol. Sixty-three mice in the second group received s.c. 0.1 of solvent without rapamycin starting at the same age and served as a control (C-2, C-4, C-5). Once a week all mice were palpated for detection of mammary tumors appearance. The localization and the size of tumors were registered. The neoplastic masses were measured with a caliper and progressively growing masses of >3 mm in mean diameter were regarded as tumors. The mean number of palpable mammary

Table 4. Effects of rapamycin on life span and spontaneous carcinogenesis in mice.

Strain	Sex	No. of mice, C/T[a]	Age at start of treatment, mo	Drug, dose and route of treatment[b]	Effect on mean life span, %	Effect on carcinogenesis[c]	References
mTOR (Δ/Δ)	M	10/17	0	No drugs, mTOR hypomorphic model	+22%	↓	Wu et al., 2013[52]
	F	24/26			+10%	↓	
UM-HET3	M	357/134	20	In food[d]	+9%	No data	Harrison et al., 2009[3]
UM-HET3	F	289/144	20		+14%	No data	
UM-HET3	M	50/50	9		+10%	No data	Miller et al., 2011[5]
UM-HET3	F	50/50	9		+18%	No data	
HER-2/neu	F	28/30	2	1.5 mg/kg, s.c. 6 times per 4 wk	+4%	↓	Anisimov et al., 2010[4]
HER-2/neu	F	12/11	2	0.45 mg/kg, s.c. 6 times per 4 wk	0	↓	Present paper
		32/32	4		+5%	↓	
		19/18	5		+7%	↓	
129/Sv	F	31/35	2	1.5 mg/kg, s.c.	+4%	↓	Anisimov et al., 2011[6]
C57BL/6	M	10/10	22–24	4 mg/kg b.w., i.p. bidaily for 6 wk	Increase	No data	Chen et al., 2009[2]
C57BL/6J	M	20/20	4	In food	Increase	↓	Neff et al., 2013[13]
	M	21/21	13				
	M	27/27	20–22				
C57BL/6Nia	M	44/45	19	In food	No effect	↓	Zhang et al., 2013[12]
	F	43/45			Increase	↓	
Ndufs4$^{-/-}$	M	No data	20 d	8 mg/kg i.p. e.o.d.	+25%	No data	Johnson et al., 2013[14]
	F				+38%		
	M+F			8 mg/kg i.p. daily	+100%	No data	
C57BL/6J *p53*$^{+/-}$	M	38/37	<5	1.5 mg/kg, d.w.	+28%	↓	Komarova et al., 2012[10]
C57BL/6J *p53*$^{+/-}$	M	38/37	>5	1.5 mg/kg, d.w.	+10%	↓	Komarova et al., 2012[10] and Comas et al., 2012[9]
C57BL/6J *p53*$^{-/-}$	M	17/21	2	0.5 mg/kg, p.o. × 5 d; break 9 d	+30%	↓	
Rb1$^{+/-}$	M	97/98	2–3	14 mg/kg, p.o. or or in food	+14%	↓	Livi et al., 2013[11]
	F		2–3		+9%	↓	
C57BL/6J *Bmal1*$^{-/-}$	M+F	73/31	3.3	0.5 mg/kg d.w.	+50%	No data	Khapre et al., 2014[53]
129Sv-C57BL/6J Lmna$^{-/-}$	M+F	23/23	1	In food	+23%	No data	Ramos et al.[8]
		11/11	1	8 mg/kg i.p. e.o.d.	+57%		
		9/11	1	8 mg/kg i.p. weekly	+53%		
C57BL/6J Lmna$^{-/-}$	M/F	11/11	1	8 mg/kg i.p. e.o.d.	+56%		

[a]C/T, Control/Treatment; bd.w., with drinking water; s.c., subcutaneously; i.p., intraperitoneally; p.o. (per os), gavage; e.o.d., every other day; [c]↑, increases; ↓, decreases; dIn food, around 14 mg/kg encapsulated rapamycin with food.

carcinomas/mouse was calculated as the cumulative number of incident tumors/number of tumor-bearing mice. The number of mice with metastases into lungs was also registered. Animals were weighed once a month and were observed throughout their lifespan.[4,51]

Pathomorphological examination

All animals were autopsied. All tumors, as well as the tissues and organs with suspected tumor development were excised, fixed in 10% buffered formalin and embedded into paraffin. Five micrometer histological sections were stained with hematoxylin and eosine and were microscopically examined. Tumors were classified according to the International Agency for Research on Cancer recommendations.

Statistics

Experimental results were statistically processed by the methods of variation statistics with the use of STATGRAPH statistic program kit as previously described.[4,43] The significance of the discrepancies was defined according to the Student *t* criterion,

Fischer exact method, χ^2, non-parametric Wilcoxon–Mann–Whitney, and Friedman RM Anova on Ranks. The Student–Newman–Keuls method was used for all pairwise multiple comparisons. Coefficient of correlation was estimated by the Spearman method. Differences in tumor incidence were evaluated by the Mantel–Haenszel log-rank test.

For experimental group the Cox regression model was used to estimate relative risk of death and tumor development under the treatment compared with the control group: $h(t,z) = h_0(t) \exp(z\beta)$, where $h(t,z)$ and $h_0(t)$ denote the conditional hazard and baseline hazard rates, respectively, β is the unknown parameter for treatment group, and z takes values 0 and 1, being an indicator variable for two samples—the control and treatment group.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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