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Original Paper

Radioimmunotherapy of Human Glioma Xenografts in Nude Mice by Indium-111 Labelled Internalising Monoclonal Antibody

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The potential of ^{111}Indium (^{111}In)-labelled internalising anti-integrin α3 antibody GA17 in the radioimmunotherapy of human glioblastoma xenografts in nude mice was investigated. A radioisotope retention assay showed a rapid release of radioiodine from the glioblastoma cells after the binding of ^{125}I-GA17, whilst ^{111}In-GA17 was retained in the cells for a longer time period. The glioblastoma xenografts showed a high and prolonged uptake of ^{111}In-GA17, and tumour uptake of ^{125}I-GA17 was lower and decreased with time. In the mice which received two injections of 18.5 MBq of ^{111}In-GA17, the growth of the subcutaneous tumour was significantly suppressed compared with the untreated group and mice injected with an ^{111}In-labelled control antibody. These results indicate that GA17 was internalised into the glioblastoma cells and that ^{111}In was retained within the cancer cells. The injection of a high-dose of ^{111}In-GA17 can suppress the growth of tumour xenografts in nude mice. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: radioimmunotherapy, glioblastoma, internalising antibody, Indium-111
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INTRODUCTION

INDIUM-111 (^{111}In), WHICH emits γ-photons, with energy ranging from 171 to 245 keV, is suitable for scintigraphic imaging and has been widely used for tumour imaging coupled with an anticancer antibody and various tumour-seeking peptides [1, 2]. In addition, ^{111}In emits internal conversion and Auger electrons of various energy levels. Theoretically, these electrons are toxic if the target is located within the effective length of the electrons [3]. In the present study, the potential of an ^{111}In-labelled internalising anti-integrin α3 monoclonal antibody (MAb) in the radioimmunotherapy of human glioblastoma xenografts in nude mice was investigated.

MATERIALS AND METHODS

Human cancer cell line

The human glioblastoma cells U87MG (American Type Culture Collection, Rockville, Maryland, U.S.A.) were grown in RPMI1640 medium supplemented with 10% fetal calf serum (FCS) and 0.03% L-glutamine.

Monoclonal antibodies and radiolabelling

The murine IgG_1 MAb GA17 was originally produced as a glioma-specific antibody by immunising mice with human glioblastoma cells [4], but was later found to be specific for human integrin α3, which was proved by amino acid sequence analysis of the affinity-purified protein and by the reactivity of the immunoprecipitated protein with anti-integrin α3 antibody (data not shown). The murine IgG_1 MAb F33-104, reactive with carcinoembryonic antigen, was used as an isotype-matched control antibody [5].

GA17 was radio-iodinated using the chloramine-T method [6]. Purified antibodies (50 μg) in 0.3 M phosphate buffer, pH 7.5, and ^{125}I (11.1 MBq, DuPont/NEN, Wilmington, Delaware, U.S.A.) were mixed with 2.5 μg of chloramine-T. After 5 min, radiolabelled antibodies were separated from free iodine through PD-10 gel chromatography (Pharmacia LKB Biotechnology, Uppsala, Sweden). The specific activity of ^{125}I-GA17 was 85.1 MBq/mg.

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The antibodies were labelled with ^{111}In using 1-(4-isothiocyanatobenzyl) ethylenediamine-N,N,N′,N′-tetraacetic acid (SCN-Bz-EDTA, Dojindo Co., Kumamoto, Japan) as a bifunctional chelating agent as previously described [7]. Briefly, antibody solutions in 0.05 M borate-buffered saline, pH 8.5, were mixed with SCN-Bz-EDTA dissolved in dimethylformamide with the mixing molar ratio of 1:5, and incubated overnight at 30°C. The unconjugated SCN-Bz-EDTA was removed by applying the mixture to PD-10 column using 20 mM 2 N-morpholino ethanesulpholic acid-buffered saline, pH 6.0, as an eluant. The conjugation ratios of the antibodies were calculated to be 3.08 for GA17 and 1.58 for F33-104. Conjugated antibodies (400 μg) were mixed with 148–185 MBq of ^{111}In-acetate, which was prepared by adding 200 μl of 1.75 M HCl and 800 μl of 1 M sodium acetate to 1 ml of ^{111}In-chloride (Nihon Mediphysics, Nishinomiya, Japan). Labelling efficiency ranged from 85 to 95%, resulting in specific activities of ^{111}In-antibodies ranging from 333 to 390 MBq/mg.

The immunoreactivities of radiolabelled GA17, measured according to the method of Lindmo and colleagues [8], were more than 80% for both ^{125}I- and ^{111}In-labelled GA17.

Radionuclide retention assay and acid-wash assay

A radionuclide retention assay was performed according to the method of Naruki and colleagues [9]. Forty million U87MG cells were incubated with 0.5 μg of ^{125}I- or ^{111}In-labelled GA17 in 1 ml of RPMI1640 medium with 10% FCS for 1 h at room temperature. Unbound radioactivity was removed by washing, and the cells were resuspended in individual tubes at a concentration of 2×10^6 cells/1 ml of RPMI1640 with 10% FCS. Tubes were incubated at 37°C. Cells were separated from the supernatant immediately prior to incubation and after 1, 3, 6, and 21 h of incubation by centrifugation. The cell pellets were washed with cold phosphate-buffered-saline (PBS), and the radioactivity of the pellets was measured. Supernatants were mixed with 1 ml of 10% trichloroacetic acid (TCA) and then divided into TCA-precipitable and TCA-nonprecipitable fractions. The radioactivities of both fractions were measured along with that of the cell pellets. Data are expressed as percentages of the radioactivity in each fraction.

Acid-wash assay was performed to determine whether radiolabelled antibodies are located on the cell surface or moved inside the cells. After incubation of ^{111}In-labelled GA17 (100 000 cpm in 1 ml of RPMI1640 medium with 10% FCS) with U87MG cells plated in 35 mm dish for 1 h at room temperature, cells were washed and added with fresh RPMI 1640 with 10% FCS. After 0, 1, 3, 6, and 23 h of incubation at 37°C, cells were washed and 1 ml of 0.05 M Glycine-HCl buffer, pH 2.8, with 0.1 M NaCl was added and incubated for 15 min at room temperature to detach antibodies from the antigen on the cell surface. After collecting the supernatant, cells were detached by incubation with 0.05% trypsin/0.02% EDTA solution for 15 min. Cells were collected and the radioactivities of the supernatant (acid-sensitive fraction) and the cells (acid-resistant fraction) were counted.

In vivo *biodistribution study*

Four million cultured U87MG cells were subcutaneously injected into the left flank of 6-weeks old female BALB/c nude mice. After 2 weeks, tumours of approximately 500 mg weight were formed.

Tumour-bearing nude mice were injected into the tail vein with a mixture of 37 kBq of ^{125}I-GA17 and 37 kBq of ^{111}In-GA17. The protein dose was adjusted to 10 μg per mouse by the addition of unlabelled GA17. At 24, 48, and 96 h after injection, groups of mice ($n=9$ for 24 and 96 h, $n=8$ for 48 h) were sacrificed by ether inhalation, their organs were removed and weighed, and the radioactivity was measured. The data are expressed both as percentages of injected dose per gram of tissue normalised to a 20 g weight mouse and as tumour-to-normal tissue ratios. For comparison, the biodistribution of ^{111}In-F33-104 antibody was also determined 48 h after injection ($n=4$). Localisation indices were also obtained; they were derived from the tumour-to-blood ratios of the specific antibody divided by those of the control antibody. Statistical analyses were done by the analysis of variance with Fisher's PSLD test.

All animal experiments were carried out in accordance with the Japanese guidelines regarding animal care and handling.

Radioimmunotherapy

10 days after receiving a subcutaneous injection of U87MG cells, mice of the treated group ($n=6$) were administered an intraperitoneal injection of 18.5 MBq of ^{111}In-GA17 (50 μg). One week later, these mice received another injection of 18.5 MBq of ^{111}In-GA17. To administer a large volume of acidic injectate for a therapeutic dose, the intraperitoneal route was selected instead of the intravenous route. The size of the subcutaneous tumours was followed for 17 days, and was compared with those of untreated mice ($n=6$).

The second experiment compared the therapeutic effects of the specific antibody and control antibody. 10 and 17 days after a subcutaneous injection of U87MG cells, groups of mice received 18.5 MBq of ^{111}In-GA17 ($n=6$), 18.5 MBq of control antibody ($n=6$), or PBS ($n=7$). Tumour size was followed for 20 days.

The data obtained are expressed as relative tumour sizes, where the tumour volume at a given time point was divided by that at the start of therapy. Statistical analyses were done using the unpaired *t*-test, comparing the time required for the tumour to grow to 10 times as large as the initial volume.

RESULTS

Fate of ^{125}I and ^{111}In after binding

As shown in Figure 1, after the binding of ^{125}I-labelled GA17 to U87MG cells, the cell-associated radioactivity steadily decreased with time at 37°C, to 64% after 21 h, with increasing radioactivity in the supernatant, most of which was TCA-nonprecipitable (28% at 21 h), probably free iodine. In contrast, the cell-associated radioactivity of ^{111}In-GA17 was 83% at 21 h.

The results of the acid-wash assay are summarised in Figure 2. Relative radioactivity of the acid-resistant fraction increased with time (17.85% at 0 h to 49.04% at 23 h) indicating that surface-bound antibodies are internalised with time, are no longer located on the cell surface and are not affected by acid-treatment.

Differential biodistribution of ^{125}I- and ^{111}In-labelled GA17

The biodistributions data are summarised in Tables 1–3. The tumour uptake of ^{125}I-GA17 was not high on day 1 (6.73%) and decreased by nearly half on day 4 (3.13%; Table

1). In contrast, the tumour uptake of ^{111}In-GA17 was 11.77% on day 1, 15.45% on day 2 and somewhat decreased to 10.19% on day 4; the levels were always higher than those of ^{125}I-GA17 (Table 2). The blood clearance values of the two radiolabelled antibodies were almost identical, resulting in higher tumour-to-blood ratios obtained with ^{111}In-GA17 at every time point (for ^{111}In-GA17 and ^{125}I-GA17, respectively: 0.96 versus 0.62 on day 1, 1.51 versus 0.70 on day 2 ($P<0.01$), and 3.24 versus 0.95 on day 4 ($P<0.0001$)). The tumour-to-normal tissue ratios in other organs were also higher with ^{111}In-GA17, except for the liver. The accumulation of the ^{111}In-control F33-104 antibody in tumours was very low, resulting in the localisation index of 2.70 on day 2.

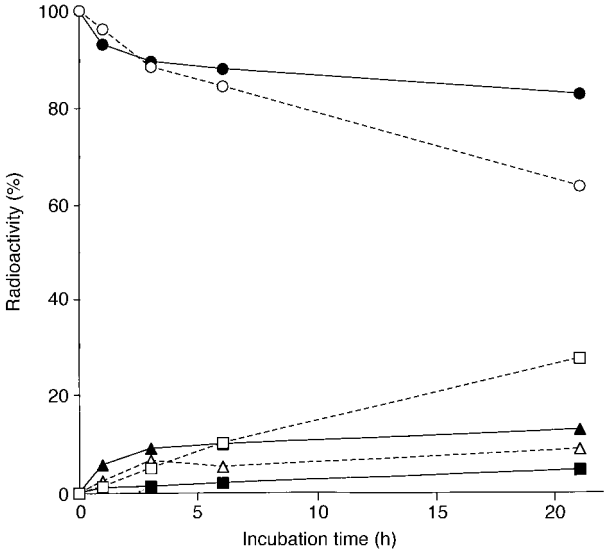

Figure 1. Radionuclide retention assay. Per cent radioactivity of each component was plotted against incubation time. (●○) cell-associated radioactivity, (▲△) TCA-precipitable radioactivity of the supernatant, (■□) TCA-nonprecipitable radioactivity of the supernatant. (●▲■) ^{111}In-GA17, (○△□) ^{125}I-GA17.

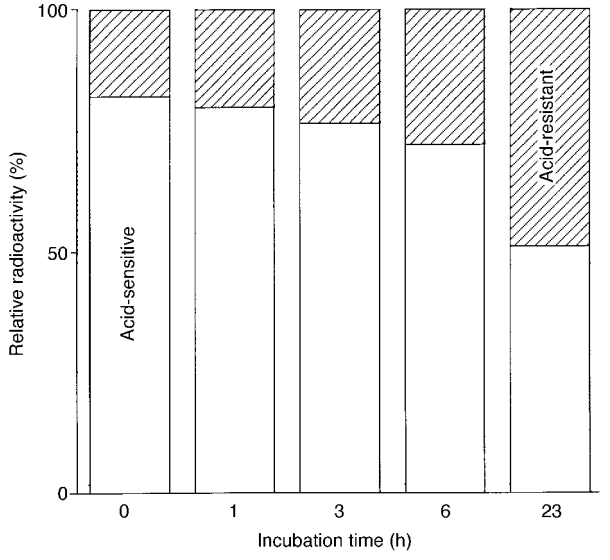

Figure 2. Acid-wash assay. Per cent radioactivity of acid-sensitive and acid-resistant fraction at each time point is illustrated.

Radioimmunotherapy

All mice tolerated the two injections of 18.5 MBq of ^{111}In-antibody (total 37 MBq). Figure 3a shows the growth curves of the subcutaneous tumour xenografts of the treated and untreated mice. Although no mouse showed a reduction in tumour size, the intraperitoneal injection of ^{111}In-GA17 significantly suppressed growth ($P<0.01$, compared with the nontreated group). As shown in Figure 3b, the growth of tumours of mice treated with ^{111}In-GA17 was also sig-

Table 1. *Biodistribution of ^{125}In-labelled GA*17 *in nude mice bearing human malignant glioma xenografts*

	Day 1 ($n=9$)	Day 2 ($n=8$)	Day 4 ($n=9$)
Blood	10.94±1.35*	9.58±1.04	3.77±2.33
Liver	3.60±1.08	2.81±0.65	1.01±0.64
Kidney	3.09±0.37	2.65±0.26	1.06±0.61
Intestine	0.96±0.10	0.86±0.09	0.37±0.20
Stomach	1.67±0.36	1.41±0.56	0.68±0.25
Spleen	2.13±0.55	2.01±0.46	0.74±0.48
Lung	4.55±0.59	3.93±0.54	1.63±0.92
Muscle	0.86±0.11	0.78±0.14	0.34±0.19
Bone	1.30±0.21	1.14±0.23	0.48±0.29
Tumour	6.73±1.10	6.61±1.22	3.13±1.41

*Mean ± standard deviation (S.D.) of per cent injected dose per gram tissue.

Table 2. *Biodistribution of ^{111}In-labelled GA*17 *in nude mice bearing human malignant glioma xenografts*

	Day 1 ($n=9$)	Day 2 ($n=8$)	Day 4 ($n=9$)
Blood	12.07±1.53*	10.30±1.03	3.97±2.44
Liver	10.77±1.82†	8.26±1.85†	5.41±1.07†
Kidney	5.16±0.39†	4.31±0.22†	2.27±0.68†
Intestine	1.55±0.13†	1.33±0.16†	0.68±0.23‡
Stomach	0.87±0.21†	0.80±0.14‡	0.42±0.19
Spleen	3.69±0.76†	3.46±0.62†	1.96±0.63†
Lung	5.16±0.64	4.52±0.57	1.99±1.01
Muscle	0.93±0.11	0.82±0.13	0.39±0.19
Bone	1.90±0.35‡	1.83±0.31†	1.05±0.39‡
Tumour	11.77±1.52†	15.45±1.81†	10.19±3.09†

*Mean ± standard deviation (S.D.) of injected dose per gram tissue. †$P<0.0001$ compared with ^{125}I-labelled GA17. ‡$P<0.0005$ compared with ^{125}I-labelled GA17.

Table 3. *Biodistribution of ^{111}In-labelled F*33-104 *in nude mice bearing human malignant glioma xenografts*

	Day 2 ($n=4$)
Blood	9.16±1.11*
Liver	5.88±1.23
Kidney	3.72±0.35
Intestine	1.09±0.14
Stomach	0.75±0.08
Spleen	2.94±0.63
Lung	3.37±1.07
Muscle	0.88±0.14
Bone	1.36±0.28
Tumour	5.10±0.71

*Mean ± standard deviation (S.D.) of per cent injected dose per gram tissue.

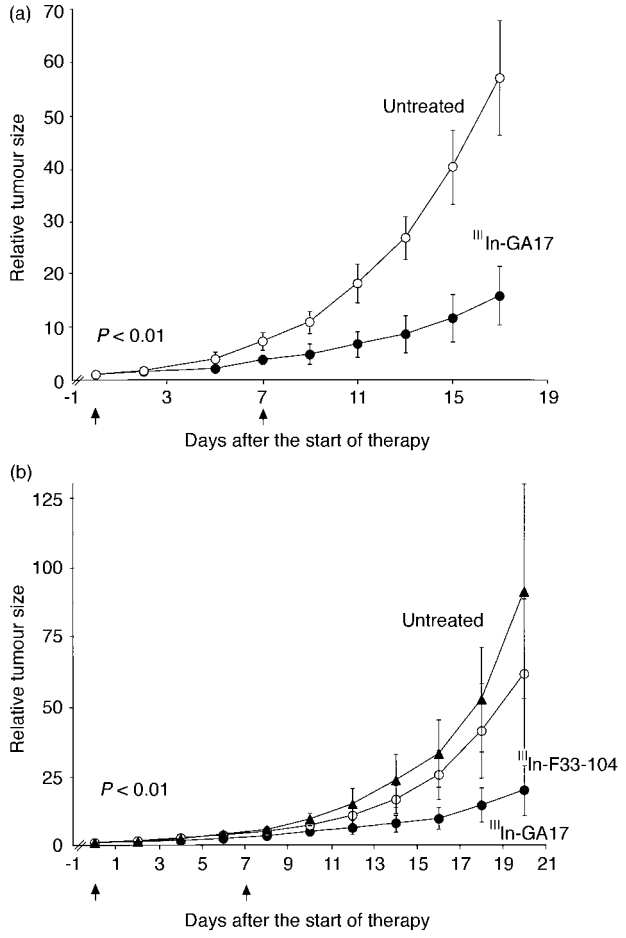

Figure 3. Growth curves of subcutaneous tumour xenografts of treated and untreated mice. (a) (●) Mice received two injections of ^{111}In-GA17. (○) Untreated mice. Arrows indicate the time of i.p. injection of ^{111}In-GA17. Data are expressed as mean ± S.D. of relative tumour size. (b) (●) Mice received two injections of ^{111}In-GA17. (○) Mice received two injections of ^{111}In-F33-104. (▲) Untreated mice. Arrows indicate the time of i.p. injection of ^{111}In-antibody. Data are expressed as mean ± S.D. of relative tumour size.

nificantly suppressed compared with those of the untreated group and mice that received ^{111}In-F33-104 ($P<0.01$). No significant difference was observed between ^{111}In-F33-104 and control groups ($P=0.1212$) although the growth of the tumours of ^{111}In-F33-104-treated mice was somewhat slower.

DISCUSSION

For targeted cancer therapy using radiolabelled compounds, radioisotopes emitting β-particles have been mainly used as radiolabels [10, 11]. The use of α-particles and Auger electrons, which are more toxic to cancer cells, has also been investigated for unsealed radiotherapy [12, 13]. Since the particle range of Auger electrons is very short (less than 10 μm), they should be located close to or make contact with the target, i.e. DNA strands, in order to exert their toxic effect. Most investigations of Auger emitters have, therefore, been conducted using DNA-seeking agents such as ^{125}I-iododeoxyuridine and ^{125}I-oestrogen [14, 15].

^{125}I-labelled internalising antibodies have recently been used for the therapy of experimental tumours and cancer patients, with the expectation that the Auger effect from the ^{125}I located in the cytoplasm will take place. Welt and colleagues reported encouraging results in a phase I/II clinical study with advanced colon cancer patients; an injection of a high-dose of ^{125}I-labelled antibody showed modest antitumour activity with no bone marrow toxicity [16]. Behr and colleagues showed, in an animal model, the superior antitumour effect of ^{125}I-labelled internalising antibody compared with that of its ^{131}I-labelled counterpart [17].

Indium-111, which has been used mainly in diagnostic nuclear medicine, is also known to emit conversion and Auger electrons and is a candidate for therapeutic application [3]. Various ^{111}In-labelled compounds have recently been investigated for their therapeutic potentials. Kairemo and colleagues studied ^{111}In-bleomycin as a radiochemotherapy agent [18], and Krenning and colleagues reported a case of a metastatic glucagonoma which responded to repeated injections of high-doses of ^{111}In-labelled octreotide [19, 20].

In the present study, we used an internalising anti-integrin α3 MAb as a carrier of ^{111}In. In addition to a wide variety of cancers, integrin α3 is also expressed in various normal tissues. However, the quantitative and qualitative difference in its expression in cancers may make it a favourable target of antibody-based imaging and therapy [21]. A previous study showed that this antibody accumulated in human cancer xenografts of various histological types and also showed that in some tumours, antibodies were internalised after binding to cancer cells [21]. In the present *in vitro* and *in vivo* studies, the internalisation of radiolabelled GA17 after binding to human glioblastoma cells and the prolonged retention of ^{111}In-metabolites within the cancer cells were demonstrated. The radioimmunotherapy experiment showed that the repeated administration of ^{111}In-GA17 had a significant therapeutic effect on the subcutaneous human glioma xenografts and indicated that ^{111}In-labelled antibody can be used as a radioimmunotherapy agent. This therapeutic effect, although not very strong, is thought to be due to the two kinds of electrons emitted by ^{111}In, not by the γ-photons used for imaging. Auger electrons emitted by ^{111}In have the energy level of 0.5–25 keV and the particle range of 0.02–10 μm. Therefore, ^{111}In should be located very close to the target, i.e. DNA. This is not possible for radiolabelled antibodies which are not internalised. When radiolabelled antibodies are internalised, they are metabolised and, in the case of radioiodinated antibody, they are dehalogenated and the free iodine will leave the cancer cells. In the case of ^{111}In-labelled antibodies, ^{111}In- metabolites are retained within the cancer cells, as reported previously and also shown in the present study by the radionuclide retention assay and acid-wash assay [9, 21, 22]. Although the exact intracellular localisation of ^{111}In-metabolites is unclear (probably mostly cytoplasmic/lysosomal according to the Percoll gradient fractionation study of cell organelles by Press and colleagues [22]), it is possible that some ^{111}In-metabolites are located very close to the nucleus, and their Auger electrons can be toxic. Conversion electrons emitted by ^{111}In have higher energy (111–245 keV) and a longer particle range (200–500 m) and can be toxic even when located distant from the nucleus. A study with two injections of unlabelled GA17 (50 μg) showed no significant growth retardation effect (data not shown).

Faraggi and colleagues reported the results of a mathematical analysis of the influence of the subcellular localisation of radioactivity on the dose-rate delivered to the cell nucleus [23]. According to their calculation for an ^{111}In-compound,

cytoplasmic localisation (without nuclear localisation) of radioactivity gave a 1.21-times higher dose-rate than membrane localisation. If the radiolabelled metabolites of internalised antibody have free access to the nucleic compartment, it will give a 7.14-times higher dose-rate. In the present case, it seems that the basic therapeutic effect may come from the higher energy conversion electrons, and that the internalisation of the ^{111}In-antibody had additional irradiation effect due to Auger electrons located close to the nucleus, although the degree of this contribution remains to be determined.

A minimal growth suppression effect, although not significant, was seen with the mice treated with ^{111}In-labelled control antibody. It may be because of a low number of mice in each group. It also may be possible that conversion electrons with longer effective length might exert some effect after injection of a high-dose of ^{111}In-labelled control antibody.

An additional point to consider is that the distribution of radioactivity within the tumour should be diffuse, hopefully homogeneous, when low-energy electrons are used which cannot cover multiple cell layers. An autoradiographical study showed that the intratumoral distribution of ^{111}In-GA17 was diffuse, although not completely homogeneous (data not shown). Smaller-sized lesions, such as metastases, will be better targets of therapy.

In conclusion, the present investigation showed that anti-integrin α3 antibody GA17 was internalised into glioblastoma cells, and the ^{111}In-metabolite formed was retained within the cells *in vitro* and also retained within the glioblastoma xenografts *in vivo*. Two injections of a high-dose of ^{111}In-GA17 can suppress the growth of glioblastoma xenografts in nude mice. ^{111}In-antibodies have potential for use in the radioimmunotherapy of cancers.

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