Research Article

A method to induce tumor marker release

Abstract

Objective: Tumor markers are considered unreliable because their levels are low and detection methods are not sufficiently sensitive and specific. Furthermore, there is no clear correlation with tumor size, because the biological characteristics of tumor markers remain largely unknown. We previously reported a reliable tumor marker combination assay in the journal Cancer in 1994 using 3 types of marker, tumor-specific, tumor- associated and growth-related makers. Here, we report a new method to induce tumor marker release and discuss related clinical applications, especially, for cancers without specific tumor markers.

Methods: The human gastric cancer line　MKN-45 (20million cells) was transplanted into nude mice. On days 20 and 27 after tumor cell transplantation tumor, 2500 IU vitamin A (retinoic acid) was administered intramuscularly. After 3 hours, the tumor bearing nude mice were treated at 40°C for 20 minutes using an infrared red ray sauna bath to induce tumor marker release. Carcinoembryonic antigen (CEA) was measured at 0, 6, 24, and 48 hours. The same doses of sodium bicarbonate and palmitic acid were used as controls. We examined the correlation of cancer stage and tumor marker parameters, including the maximum CEA value (CEAmax), the maximum differences in CEA levels (ΔCEA: range of tumor marker levels), average variation (ΔCEA/ h) and the expected maximum quantity (ΔCEA/h xCEAmax), after combined treatment with vitamin A and hyperthermia in nude mice and human.

Results: Tumor markers have been considered substances released by broken cancer cells. However, we observed that tumor marker release is induced by hyperthermia treatment with vitamin A and is inhibited by treatment with actinomycin D or cycloheximide via CEA mRNA. Tumor marker release might be related to tumor markers induced by mRNA synthesis. We designed a method to estimate the correlation between the presence of tumors and tumor size, using tumor-marker parameters.

Conclusion: We can estimate the tumor size with the maximum value(Vmax), maximum differences in value (Δmax), the average variation (Δmax/h) and expected maximum quantity (ΔmaxxVmax) based on the release of tumor markers by combining vitamin A and hyperthermia treatment even if tumor lacks specific tumor marker.

Keywords: induction of tumor marker, maximum difference in value (Δmax), maximum value (Vmax), the average variation (Vmax/h), expected maximum quantity (VmaxxΔmax/h)

Tsuneo Kobayashi
International cancer prevention center, Japan

Correspondence: Tsuneo Kobayashi, International cancer prevention center, Chiba city, 3-21-1, Takasu, Mihamaku, Chiba city 261-0004, Japan, Tel 043-306-2611, Fax 043-279-4211, Email ft1992@vega.ocn.ne.jp

Introduction

Although, Kitamura[1] reported that the maximum range variation for carcinoembryonic antigen (CEA) levels in a healthy body is narrow for many years.[1] We observed that serum tumor markers are not constant in any cancer case. To date, the induction of tumor marker release (TMR) has never been applied to patients during an initial examination in an outpatient clinic. Prior to patient examination, we verified the utility of this method in animal and tissue culture experiments.

Basic experiments

a) Long-term induction of tumor marker release (TMR)[2]

We examined the correlation between tumor size and tumor marker parameters including the maximum level of the tumor marker CEA (CEAmax), the change in the CEA value (ΔCEA), the rate of change of the CEA value (ΔCEA/h), and ΔCEA/h x CEAmax. We revealed long-term (Figure 1) and short-term (Figure 2) TMR induction; CEA release was induced by vitamin A (Chocola®, retinoic acid 25 ～125μM) and hyperthermia (40°C for 20min). CEA was assayed with an enzyme immunoassay using a CEA-EIA kit (Hoffman-Roche

Co., Basel, Switzerland)

b) Short-term induction of TMR (Figure 2)

TMR was characterized by short-term and long-term biphasic induction reactions. Because short-term TMR induction is difficult to use in clinical practice, long-term TMR induction (1 to 2days) is conventionally used.

c) This phenomenon was unchanged. Even when the mouse model changed from CD-1/nu.nu to Balb-nu/nu; similar results were obtained.

d) TMR was induced 20 and day 27days after transplantation. Correlation of tumor size with the following parameters was assessed: CEAmax, ΔCEA, ΔCEA/h, and ΔCEA/hxCEAmax. A good correlation was obtained.

e) Study of cultured cells[3]

CEA-producing human cultured cancer cells (MKN-45) were treated with vitamin A (retinoic acid, 75 to 125μM). After treatment, cell growth was slightly inhibited, and intracellular and extracellular CEA levels increased (Figure 3).
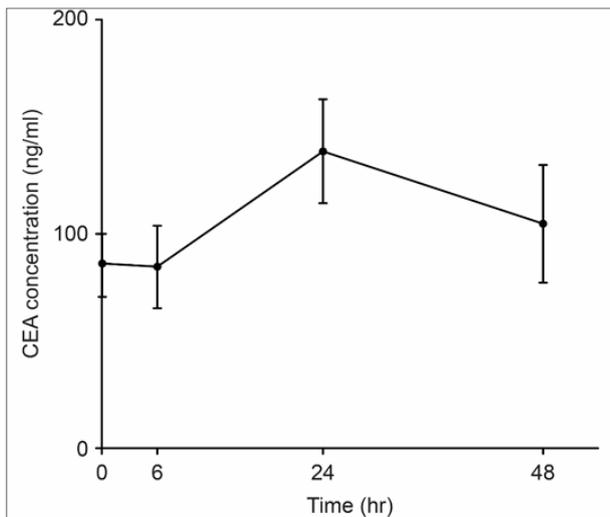
Figure 1 TMR induction was performed out on day of 16 after transplantation of MKN-45 (20million cells) in the long term. Three hours after intramuscular injection of vitamin A (2500 IU), hyperthermia treatment was performed at 40°C for 20minutes. The blood was withdrawn from the orbital vein at 0, 6, 24 and 48 hours. CEA was assayed by the CEA-EIA kit (Abbott, Co. Ltd).
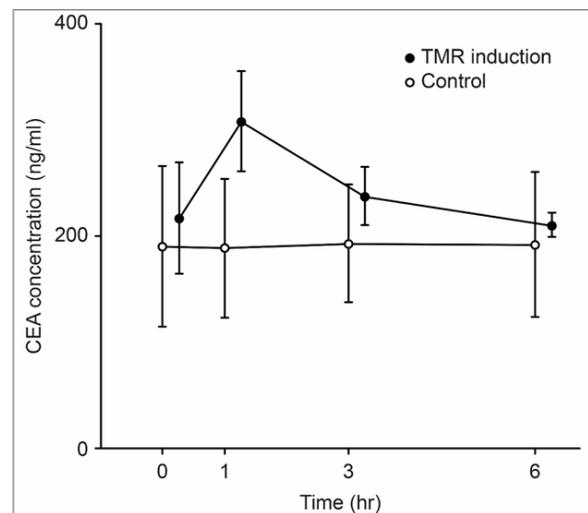

Figure 2 TMR induction was performed out on day of 16 after transplantation of MKN-45 (20million cells) in the short term. Three hours after intra-muscular injection of vitamin A (2500IU), hyperthermia treatment was added at 40°C for 20minutes. The blood was collected from orbital vein after 0, 1, 3, and 6 hours. CEA was assayed by the CEA-EIA kit (Abbott, Co. Ltd). In the control, the same amounts of HCO and palmitic acid were injected.
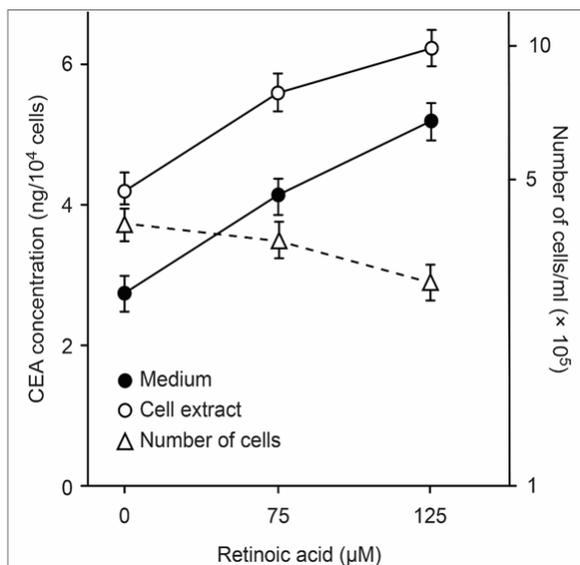
by treatment with vitamin A and hyperthermia and inhibited by treatment with *actinomycin* D (1ng/mL) and cycloheximide (0.2μg/mL), which are protein synthesis inhibitors. The open white columns indicate the control group. The solid black columns indicate the group treated with vitamin A and hyperthermia. The columns with the slanted lines indicate the group treated with vitamin A, hyperthermia and *actinomycin* D. The columns with vertical lines indicate the group treated with vitamin A, hyperthermia, and cycloheximide. As shown in Figure 5, CEA release was induced by combined treatment with vitamin A and hyperthermia. Because mRNA inhibitors prevent this reaction, the protein synthesis observed was newly induced. There was a trend toward increased CEA release over time after stimulation by vitamin A and hyperthermia. CEA release was consistently inhibited by treatment with *actinomycin* D or *cycloheximide*. From these studies, even for small tumors, we can estimate the size of hidden tumors in the body by utilizing tumor marker parameters such as the maximum difference in a value(Δmax), the maximum value(Vmax), the average variation(Δmax/h) and the expected maximum quantity(Δmax/h xVmax). Therefore, we performed a tumor marker induction experiment with specific patients (Table 1). The times of the final blood collection and the number of patients for each blood sampling are shown in Table 2.

Figure 3 Dose-dependency of the CEA increase induced by retinoic acid in MKN-45 cells. The cells were incubated in the presence of retinoic acid at various concentrations for 2 days. The CEA content in the medium (●) and in cell extract (○) and the number of cells (△) was measured by the methods described in Section 2.

f) Next, MKN-45 cells were treated with vitamin A (125μM retinoic acid) combined with hyperthermia (40°C for 30 min.) to investigate proliferation over time and changes in intracellular and extracellular CEA levels (Figure 4). Treatment with vitamin A and hyperthermia increased intracellular and extracellular CEA levels. CEA was induced by vitamin A, and further treatment with hyperthermia synergistically induced CEA.

g) Cultured MKN-45 cells (3x10⁵/tube) were used to examine whether CEA released from CEA-producing tumors is induced

Methods for inducing TMR in humans[4]

A total of 138 patients presented at a holistic medical outpatient clinic and received a histopathological definitive diagnosis. The tumor size was 1 to 2 cm in diameter for cancer stage G_1, 2 to 5 cm in diameter for cancer stage for G_2, and 5 cm or greater in diameter for cancer stage G_3, which was classified as advanced cancer. Stage G_1, G_2 and G_3 numbers included 61, 37 and 40 cases, respectively. As a control, the induction of TMR was studied in 11 patients without cancer and in 4 healthy subjects. Tumor marker release was examined. Table 1 shows the types of cancer.

Table 1 Diagnosis subject who have various cancer, were utilized in TMR experiment

Carcinoma	No. of patients
Breast	25
Stomach	21
Colon	15
Rectum	10
Uterus	8
Lung	8
Larynx	3
Liver	5
Kidney	4
Nose	2
Esophagus	2
Malognant Lymphoma	2
Hodgkin disease	2
Seminoma	4
Leukemia	2
Miscellaneous	25
Controls	
Healthy individuals	4
Non-cancer patient	11

Table 2 Times of final blood collection and number of patients for each blood sampling

Induction period after hyperthermia (h)	Number of cases							
	Four markers				Five markers			
	G_1	G_2	G_3	Total	G_1	G_2	G_3	Total
6	11	11	9	31	1	0	2	3
8	8	1	5	14	5	0	3	8
24	20	9	4	33	12	5	4	21
48	22	16	22	60	12	12	9	33
Total	61	37	40	138	30	17	18	65

CEA, ferritin (FT), AFP, and FT/Fe in the case of four markers

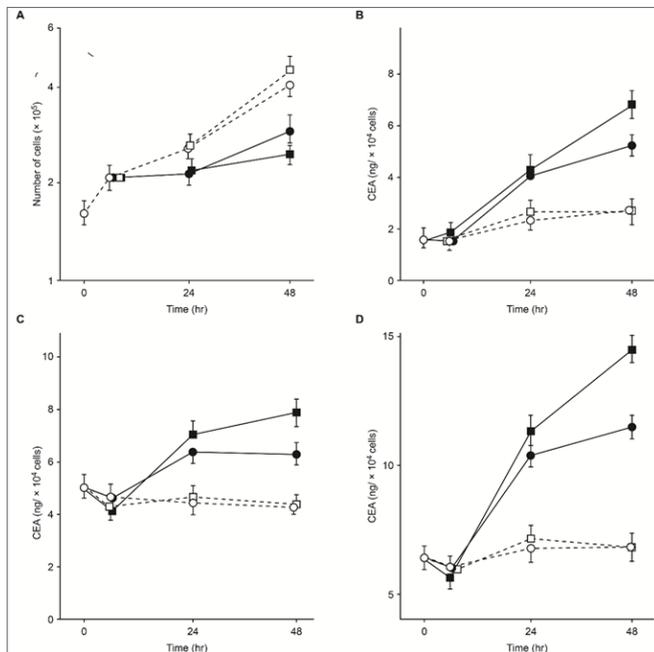CEA, ferritin (FT), AFP, FT/Fe, and sialic acid in the case of five markers
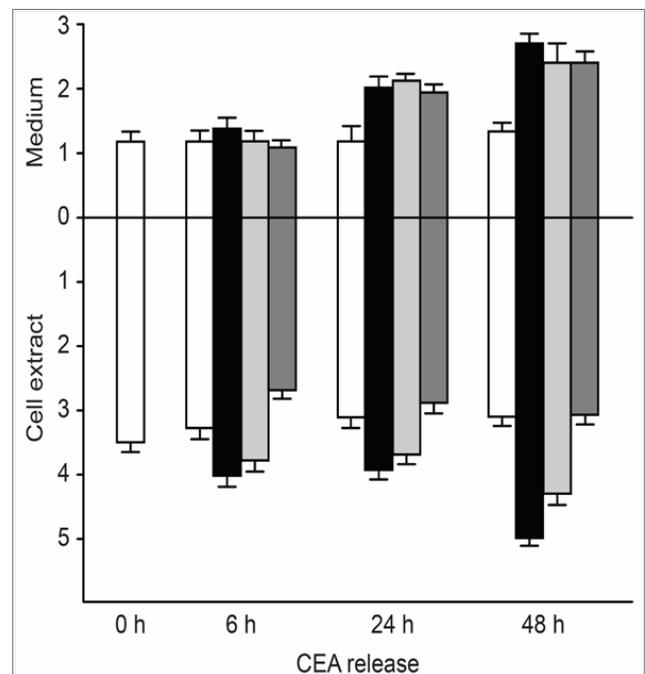

Figure 5 Effects of mRNA transcription or protein synthesis inhibitors on retinoic acid and hyperthermia-mediated increases in CEA content in MKN-45 cells. Cells were simultaneously exposed to 75µM retinoic acid and either actinomycin D (1ng/ml) or cyclohexamide (0.2µg/ml) followed by hyperthermia (40°C, 30min). After 6, 24 and 48h. cells and media were collected and analyzed for CEA. (□)Control: (■) retinoic acid + hyperthermia: ( ) retinoic acid + hyperthermia + actinomycinD: ( ) retinoic acid + hyperthermia + cyclohexamide.

Determination of tumor markers

CEA levels were determined by enzyme immunoassay with kits obtained from Abbott Co., Ltd. The cut-off value for CEA(Vmax) in stage G_1 and the cut-off value of Δmax (hereafter referred to as Δ in Tables 3–5) determined as the difference between the maximum and minimum values, were found to be 4.4ng/ml and 0.9ng/ml, respectively. The cut-off value of Δmax per hour (Δmax/h) and the cut-off value of (Δmax/h)xVmax were calculated to be 0.1ng/ml.h and 0.25ng²/ml².h, respectively. Values of ferritin, alpha-fetoprotein (AFP), sialic acid and the ratio of ferritin to serum iron (FT/ Fe) were

Figure 4 The time course of CEA increase induced by retinoic acid and in combination with hyperthermia in MKN-45 cells. Cells were continuously treated with or without 125µM retinoic acid in culture tubes. Then, the cells were heated at 40°C for 30min immediately after the retinoic acid was added. All of the points represent the mean value of three tubes. (○) Control. (□) hyperthermia: (●) retinoic acid: (■) retinoic acid+ hyperthermia. (A). Cell growth: (B)-(D) CEA levels.

also determined using four parameters (Δmax, Vmax, Δmax/h, and Δ (Δmax/h)xVmax). The cut-off values for each stage are shown in Table 3. To quantitatively assess ferritin levels, the SPAC-Ferritin kit (The First Radioisotope Research Institute, Tokyo, Japan) and RIA-gnost Ferritin kit (Hoechst, Frankfurt, FRG) were used. The AFP level was determined by RIA (double antibody method). Sialic acid levels were determined by enzyme assay with neuraminidase. Serum iron (Fe) was assayed with a Hitachi 705 autoanalyzer

Table 3 Cut-off values for tumor markers

Tumor marker	Cancer stage		
	G_1	G_2	G_3
CEA (ng/ml)			
Vmax	4.4	4.8	10
Δ	0.9	1	1.5
Δ/h	0.1	0.12	0.25
Δ/h × Vmax	0.25	0.35	1
FT SPAC (ng/ml)			
Vmax	M:150, F:65	190	300
Δ	20	30	40
Δ/h	0.8	1.5	4
Δ/h × Vmax	50	100	400
RIA			
Vmax	M:200, F:100	300	450
Δ	M:30, F:40	M:50, F:40	60
Δ/h	4	7	15
Δ/h × Vmax	800	2,000	5,000
AFP (ng/ml)			
Vmax	10	20	50
Δ	5	6	7
Δ/h	0.6	0.6	0.8
Δ/h × Vmax	3	4	8
Sialic acid (mg/dl)			
Vmax	66	70	73
Δ	5	7	10
Δ/h	0.5	0.65	0.8
Δ/h × Vmax	30	35	45
FT/Fe (ng/ml/µg/dl)			
SPAC	1	2	3
RIA	1.5	2.5	5

Preliminary results

The cut-off value for each of the four parameters at the G_1 level was used for each tumor marker, and the number and rate of positive cases for tumor stages (G_1, G_2 and G_3) are shown in Table 3. When the cut-off value of Δmax or Vmax at a G_1 level was used for CEA, the rates for positive cases in stages G_1, G_2 and G_3 were 64, 62 and 70%,

respectively. When the cut-off value of Δmax/h or (Δmax/h)xVmax was used, the rates for positive cases in stages G_1, G_2 and G_3 were 61, 73 and 70% respectively. When any one of the four parameters were examined using the SPAC-ferritin kit to determine ferritin level, the rates of positive cases in G_1, G_2, and G_3 were 54, 65 and 86%, respectively. When any one of the four parameters were examined using the RIA-gnost ferritin kit to determine the ferritin level, the rates for positive cases in G_1, G_2 and G_3 were 61, 65 and 84%, respectively. Thus, as the result of the determining the ferritin level with the above two ferritin kits, the rates for positive cases in stages G_1, G_2 and G_3 were 57, 65 and 85%, respectively. When any one of four parameters (Δmax, Vmax, Δmax/h, or (Δmax/h) x Vmax) were used for AFP, the rates of positive cases were 26, 22, and 43% respectively. AFP was very unreliable. When any one of these parameters was used for silic acid, the rates of positive cases in G_1, G_2 and G_3 were 77, 82 and 89%, respectively. Because the ratio of ferritin Vmax (ng/ml) divided by serum iron (Fe, µg/dl) for the same period is considered an important tumor marker, we determined the rate of positive cases before the release induction by combing vitamin A and hyperthermia (0hr). When the SPAC-ferritin kit was used, the rates for stages G_1, G_2 and G_3 were 29, 60 and 76%, respectively. When the RIA-gnost ferritin kit was used, they were 52, 59 and 84%, respectively. As a result of concurrent determinations with both kits, it was found that the rate of positive cases positively correlated with increased tumor stage.

The rates for five tumor markers are summarized in Table 5. When any of four parameters were used, the rates of two positive markers to four markers were 64, 70 and 93% in stages G_1, G_2 and G_3 respectively, suggesting a linear correlation between tumor marker level and tumor size. Among any three of five positive tumor markers, any one of 4 were 53, 65 and 94% in G_1, G_2 and G_3, respectively, suggesting the same tendency. In this regard, none of the healthy controls or the non-tumor bearing patients (except for those with elevated ferritin levels caused by liver dysfunction), showed false-positive findings for tumor markers. A cut-off value was assigned in each stage for each tumor marker (Table 3). For comparison, the rates determined using the cut-off values conventionally used before induction (at 0hr) are shown in Table 6. The positive rates per cases for any two of four markers was low, 11, 14% and 55% at level of G_1, G_2, and G_3 respectively, suggesting no correlation between the markers and tumor size. As shown in Table 6, when TMR was not induced, the correlations of the tumor marker parameters with a cancer stage from G_1 to G_3 were not confirmed.

Practical application of a method to induce TMR in patients with poorly defined cancer

A method for inducing TMR can facilitate the diagnosis of cancers that are difficult to diagnose morphologically or poorly differentiated cancers, as well as the diagnosis of cancers for which specific markers are unavailable. In fact, diagnosis can be made by the combined use of tumor marker combination assay (TMCA) that we developed.[5] In clinical practice, a 30-year-old woman was given a diagnosis of poorly differentiated ovarian cancer in K. Medical University, and underwent 2 surgical operations and 4 chemotherapy sessions. She was diagnosed as having "no recurrence" by Professor N. President of the Japan Society of Obstetrics and Gynecology. However, this patient consulted with another professor because of her poor condition and was referred and admitted to our hospital. The results of TMR are shown in Table 7.

Table 4 Determination of serum CEA, ferritin, α-fetoprotein, sialic acid, and FT/Fe in cancer patients

Tumor marker	Cancer stage			Normal subjects	Cut-off used
	G_1	G_2	G_3		
CEA					
Δ or Vmax	36/61†, (64)‡	23/37 (62)	28/40 (70)	1/15 (7)	0.9 or 4.4
Δ/h or Δ/h × Vmax	37/61 (61)	27/37 (73)	28/40 (70)	1/15 (7)	0.1 or 0.25
Either one of four	42/61 (69)	30/37 (81)	31/40 (78)	1/15 (7)	
FT SPAC-ferritin kit					
Δ or Vmax	12/28 (43)	11/20 (55)	15/21 (71)	3/10 (30)	20 or M:150, F:65
Δ/h or Δ/h × Vmax	13/28 (46)	13/20 (65)	17/21 (81)	4/10 (40)	0.8 or 50
Either one of four	15/28 (54)	13/20 (65)	18/21 (86)	4/10 (40)	
RIA-gnost FT kit					
Δ or Vmax	19/33 (58)	11/17 (65)	16/19 (84)	0/5 (0)	M:40, F:30 or M:200, F:100
Δ/h or Δ/h × Vmax	17/33 (52)	10/17 (59)	13/19 (68)	0/5 (0)	4 or 800
Either one of four	20/33 (61)	11/17 (65)	16/19 (84)	0/5 (0)	
SPAC and RIA					
Δ or Vmax	31/61 (51)	22/37 (59)	31/40 (78)	3/15 (20)	
Δ/h or Δ/h × Vmax	30/61 (49)	23/37 (62)	30/40 (75)	4/15 (27)	
Either one of four	35/61 (57)	24/37 (65)	34/40 (85)	4/15 (27)	
AFP					
Δ or Vmax	11/61 (18)	3/37 (8)	14/40 (35)	1/15 (7)	5 or 10
Δ/h or Δ/h × Vmax	12/61 (20)	7/37 (19)	11/40 (28)	0/15 (0)	0.6 or 3
Either one of four	15/61 (26)	8/37 (22)	17/40 (43)	1/15 (7)	
Sialic acid					
Δ or Vmax	22/30 (73)	14/17 (82)	15/18 (83)	0/4 (0)	5 or 66
Δ/h or Δ/h × Vmax	17/30 (57)	10/17 (59)	15/18 (83)	0/4 (0)	0.5 or 30
Either one of four	23/30 (77)	14/17 (82)	16/18 (89)	0/4 (0)	
FT/Fe					
SPAC	8/28 (29)	12/20 (60)	16/21 (76)	2/10 (20)	1
RIA	17/33 (52)	10/17 (59)	16/19 (84)	0/5 (0)	1.5
SPAC and RIA	25/61 (41)	22/37 (59)	32/40 (80)	2/15 (13)	

†No. of cases

‡Percent positive

Table 5 Comblination assay of serum CEA, FT, AFP, FT/Fe and or sialic acid in cancer patients who received TMR for tumors of various sizes

	Cancer Stage			Nonmalignment diseases	Healthy individuals
	G_1	G_2	G_3		
Any two of four markers†					
Δ or Vmax	54(33/61)‡	62(23/37)	88(35/40)	18(2/11)	0(0/4)
Δ/h or Δ/h×Vmax	56(34/61)	70(26/37)	83(33/40)	18(2/11)	0(0/4)
Either one of 4	64(39/61)	70(26/37)	93(37/40)	18(2/11)	0(0/4)
Any two of five markers§					
Δ or Vmax	73(22/30)	76(13/17)	94(17/18)	nd¶	0(0/4)

Table continued....

	Cancer Stage			Nonmalignment diseases	Healthy individuals
Δ/h or Δ/h×Vmax	67(20/30)	76(13/17)	94(17/18)	nd	0(0/4)
Either one of 4	77(22/30)	82(14/17)	94(17/18)	nd	0(0/4)
Any three of five markers§					
Δ or Vmax	50(15/30)	59(10/18)	94(17/18)	nd	0(0/4)
Δ/h or Δ/h×Vmax	47(14/30)	47(8/17)	78(14/18)	nd	0(0/4)
Either one of 4	53(16/30)	65(11/17)	94(17/18)	nd	0(0/4)

†CEP, ferritin, AFP and FT/Fe

‡No. of Cases given in parantheses

§CEA, FT, AFP, FT/Fe and or sialic acid

¶Not determined

Table 6 Combination assay of tumor marker without TMR induction

Tumor markers	% Positive						Cut-off used
	G_1		G_2		G_3		
CEA	20	(12/61)†	27	(10/37)	53	(21/40)	4.4ng/ml
FT							
SPAC	29	(8/28)	45	(9/20)	62	(13/21)	F, 65; M, 150
RIA	45	(15/33)	53	(9/17)	84	(16/19)	F, 100; M, 200 (ng/ml)
SPAC and RIA	38	(23/61)	49	(18/37)	73	(29/40)	
AFP	8	(5/61)	3	(1/37)	20	(8/40)	10ng/ml
Sialic acid	30	(9/30)	29	(5/17)	56	(10/18)	66ng/ml
FT/Fe							
SPAC	0	(0/28)	0	(0/20)	10	(2/21)	10
RIA	0	(0/33)	0	(0/17)	11	(2/19)	20
SPAC and RIA	0	(0/61)	0	(0/37)	10	(4/40)	
Any two of four markers	11	(7/61)	14	(5/37)	55	(22/40)	
Any two of five markers	30	(9/30)	29	(5/17)	94	(17/18)	
Any three of five markers	3	(1/30)	12	(2/17)	39	(7/18)	

†No. of cases is given in parentheses.

Table 7 TMR induction in the postoperative undifferentiated ovarian cancer patient

TMR	0 h	6 h	24 h	48 h	
CEA	2.1	2.2	3.1	2.2	Δ CEA (1.0), CEAmax (3.1)
Ferritin	160	170	150	130	Δ FT (40ng), FTmax (170)
FT/Fe	2.3	2.6	2.6	2	FT/Fe, max (2.6)
ALP	130	122	129	122	ALPmax (130), Δ ALP (8)
LDH	319	335	294	296	LDHmax (305), Δ LDH (41)
CA15-3	12	13	14	11	CA15-3 (14), Δ CA15-3 (3)
Fe	70	65	56	62	Fe max (56), Δ Fe (14)

Ribonuclease, 202U, NK activity (17%), T cell number (1468)

Albumin: 64%, α1-globulin: 3.5%

Application results

The TMR method was applied to this postoperative undifferentiated ovarian cancer patient.

The following data were obtained after TMR induction:

CEAmax: 3.1ng (ΔCEA,1.0ng, Δ indicates the maximum change in value within 48 hours after induction: normal range within under 0.9ng), ferritin (FTmax): 170ng(normal range under 65ng), ΔFT(40ng, normal range within 20ng), FT/Fe(max): 2.6, (G_2: normal range under 2.0. ΔFT/ Fe: 0.6, LDH: 294 (Δ41U, normal range within 30U), RNase (202U: normal range within 60-90U), alpha(α)1-globulin fraction: 3.5%(normal range within 2.2~2.6%): and albumin: 64%(normal range 64~72%) Table 7. Tumor markers were evaluated by the TMCA technique, reported in the Cancer in 1994,[5] combined with the TMR induction technique. The more polished TMCA was reported in Cancer medicine.[6] Based on the results obtained with these methods, the presence of cancer in the G_2 clinical stage was estimated. Therefore, positron emission tomography (PET) and computed tomography (CT) were performed and revealed metastasis in the right axillary lymph nodes⬜Virchow-lymph⬜node metastasis (+). The

patient was given the herbal medicine: Sun Advance® which inhibits aerobic respiration by cancer cells,[7,8] and detoxification therapy twice a week. As shown in Table 8, the patient was completely cured. As of 18 years after treatment, the patient has had no recurrence.

Table 8 The time course of the ovarian cancer patient

Date	Oct. 1999	Jul-00	Jan. 2001	Feb. 2002	Jan. 2005	Jul-15
RNase	202U	147	111	99	99	
Thymidine kinase						7.7
Albumin (%)	64	67.4	65.1	65.2	62.9	61.1
α-1 globulin (%)	3.5	2.9	3	2.9	2.9	2.6
NK activity (%)	17.8	24		66		
TS risk assessment	$TS(V,G_2)$	$TS(V,G_1)$	$TS(V,G_1)$	$TS(IV,G_0)$	$TS(IV,G_0)$	TS(III)
PET scan	(+)					

Conclusions

The presence and the size of hidden cancerous tumors can be estimated with tumor marker-release-related specific parameters with a combination treatment of vitamin A and hyperthermia.

Acknowledgements

I will express sincere gratitude to Kijima Chizuko, Dogome Miyoko, and Sugimoto Kimito.

Conflict of interest

The author declares that there is no conflict of interest.

References

1. Kitamura M, Ito S, Iimori I, et al. Normal value of the serum CEA by enzyme immunoassay. *Igaku no Ayumi.* 1980;112:519–521.

2. Kobayashi T, Kawakubo T, Jo T, et al. Elucidation of Tumor marker inducing mechanism. *Newest Medicine.* 1985;40:130–1303.

3. Kobayashi T, Dogome M, Kawakubo T. Increase in carcinoembryonic antigen release from cancer cells by combined treatment with retinoic acid and low-temperature hyperthermia. *Int J Hyperthermia.* 1990;6(4):785-92.

4. Kobayashi T. Correlation between Tumor Markers and Tumor Size. *Cancer Detect Prev.* 1987;10(1-2):81–87.

5. Kobayashi T, Kawakubo T. Prospective investigation of tumor markers and risk assessment in early cancer screening. *Cancer.* 1994;73(7):1946–1953.

6. Kobayashi T. A blood tumor marker combination assay produces high seensitivity and specificity for cancer according to the natural history of cancer. *Cancer Med.* 2018;7(3):549–556.

7. Sugimoto K. et al. The effect of the anti-tumor herb medicine Sun Advance in mice. *Proc Symp WAKAN YAKU.* 1982;15:224–227.

8. Tanimizu T, et al. New approach to Chinese herb medicine, inhibition by Chinese herb medicine" Sun Advance" of SV40 transformation in mouse cells. *Proc Symp WAKAN YAKU.* 1982;15:228–233.