induced the expansion or shrinkage of particular memory T-cell clones, concomitant with a reduced capacity to maintain fully diverse repertoires of helper T-cell memory.

Previously, we have reported that human memory CD4 T cells can be discriminated into three functionally different subsets (M1, M2, and M3) using the human stem cell-associated (HSCA)- 2 monoclonal antibody (mAb) that recognises a sialic acid-dependent epitope on the low molecular mass (~115 kDa) glycoform of CD43 (Ohara et al. 2002, Kyoizumi et al. 2004). The M1 subset consists of functionally mature cells whose CD43 expression is relatively high. The M2 subset expresses moderate levels of CD43, and responds weakly to TCR-mediated stimuli. The M3 subset exhibits relatively low levels of CD43 and is anergic to TCR-mediated stimuli, and prone to spontaneous apoptosis.

In this study, we evaluated the extent to which T-cell memory function is retained in A-bomb survivors by examining the relationships between these memory CD4 T-cell subsets, ageing, and radiation exposure.

Materials and methods

Blood donors

An A-bomb survivor cohort was randomly selected from a group of Hiroshima participants in the Adult Health Study (AHS) at the Radiation Effects Research Foundation (RERF) (Kodama et al. 1996).

For the present study, blood samples of 1132 survivors were obtained, with informed consent, from survivors who participated in the AHS between 2004 and 2008. This study protocol has been approved by the Human Investigation Committee of RERF. We excluded 216 subjects (19% in total subjects) who had been diagnosed with cancer from the current study. Cancer prevalence by dose category was 16% at <0.005 Gy, 21% at 0.005–0.5 Gy, 30% at 0.5–1.0 Gy, and 35% at ≥1.0 Gy, and tended to be higher in survivors exposed to higher doses, in accord with a recent observation in the AHS population (Kyoizumi et al. 2005). The age, gender and radiation dose of the remaining 916 survivors whose lymphocyte samples were subjected to data analysis in our study are listed in Table I. Radiation doses are based on the Dosimetry System 2002 (DS02) estimates (Cullings et al. 2006).

Table I. Age, gender, and radiation dose distribution of study population.

Dose (Gy)	Age (yrs)[b] category						
	60–69 yrs		70–79 yrs		≥80 yrs		
	Male	Female	Male	Female	Male	Female	Total
<0.005[a]	27	25	58	84	17	94	305
0.005–0.5	13	33	54	66	22	107	295
0.5–1.0	19	18	20	33	9	49	148
1.0–4.0	28	25	28	35	14	38	168
Total	87	101	160	218	62	288	916

[a]Individuals in this dose category were exposed at distances in excess of 3 km from the hypocenter, and hence received doses that are substantially equivalent to zero. [b]Age at the time of the examinations that were conducted between 2004 and 2008.

Flow cytometry

Mononuclear cell fractions separated by the Ficoll-Hypaque gradient technique were analysed by three-colour flow cytometry using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA). Fluorescein isothiocyanate (FITC)-labelled HSCA-2 mAb was prepared as described previously (Kyoizumi et al. 2004). PerCP-labelled CD4 mAb and phycoerythrin (PE)-labelled CD45-related O (CD45RO) mAb were purchased from BD-PharMingen (San Diego, CA, USA) and Caltag Laboratories (Burlingame, CA, USA), respectively. Three different memory CD4 subsets were defined: $CD45RO^+$ cells that expressed higher (M1), intermediate (M2), and lower (M3) levels of CD43. For each donor specimen, the window for the M2 subset was set in a range where CD43 level was from ½- to 2-fold of the mean CD43 intensity for $CD45RO^-$ cells, and the windows for the M1 and M3 subsets were set just to the right and left sides of the M2 window, respectively (Figure 1). Note that this method of memory CD4 T-cell subset discrimination was established in a previous study (Ohara et al. 2002) in which functional and phenotypical differences among these subsets were characterised, using a gating procedure (i.e., that involved internal standardisation of fluorescence intensities) that avoided the effects of inter-experimental variability. The percentage of cells in the range of each subset was obtained in a total CD4 T-cell population.

Data analysis

Associations of the percentage of each memory CD4 T-cell subpopulation (*percentage*) with age at time of examination (*age*), gender (*gender*), and radiation dose (*dose*) were analysed using a multiple regression model (Armitage et al. 2002). The method assumed that the percentage of each T-cell subpopulation related to each explanatory variable in a log linear manner:

$$\log(percentage = \alpha + \beta_1(age - 70) + \beta_2 gender + \beta_3 does,$$

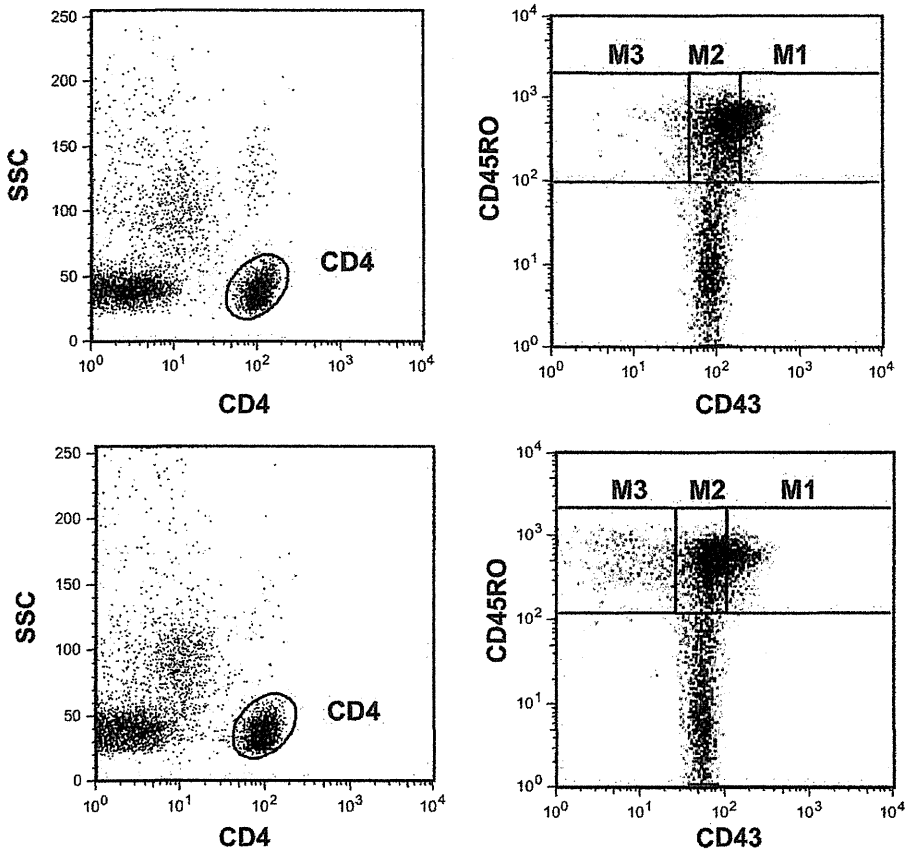


Figure 1. Flow cytometry patterns of CD4 T cells in the peripheral blood of 79-year-old females whose estimated radiation doses were zero (upper) and 0.525 Gy (lower). Peripheral blood mononuclear cells (about 2×10^5) were stained with FITC-labelled CD43 (HSCA-2) mAb, PE-labelled CD45RO mAb, and PerCP-labelled CD4 mAb. CD4 T cells were gated based on side light scattering (SSC) and CD4 intensity (left) and analysed for their expression of CD43 and CD45RO (right). Percentages of memory CD4 T-cell subsets in a total CD4 T-cell population of the unexposed were 24.1 for M1, 30.4 for M2, and 4.8 for M3, and those of the exposed were 17.2 for M1, 35.1 for M2, and 10.6 for M3.

where *gender* is an indicator of female sex, i.e., *gender* = 0 for male and *gender* = 1 for female, and *dose* is radiation dose in grays. The α is a constant term, and β_1, β_2, *and* β_3 are regression coefficients for variables to be estimated. The age term was subtracted by 70 years so that α corresponds to log-transformed percentage of CD4 T-cell subset, i.e., the subset percentage is calculated to be e^{α} (=exponential [α]), for non-irradiated males at 70 years of age. The % change of subset percentage was estimated to be $100(e^{10\beta_1}-1)$ per 10 years increment of age, and $100(e^{\beta_3}-1)$ per 1 Gy radiation dose. This regression analysis in the log linear manner was applied to evaluate the association of the percentage of memory subset within the CD4 T-cell population or CD45RO-positive memory CD4 T-cell population with age or radiation dose.

Results

Figure 1 shows the flow cytometry patterns of memory CD4 T-cell subsets within blood lymphocyte specimens of two age-matched women whose estimated exposure doses were zero and 0.525 Gy, respectively. Crude mean of percentage of each memory T-cell subset within the CD4 T-cell population was shown by age category and by dose category in Tables II and III, respectively. Table IV shows the association of the percentage of each memory T-cell subset with age and radiation dose, in terms of a multiple regression model. The percentage of memory cells (identified and enumerated by CD45RO-positivity) within the CD4 T-cell population appeared to significantly increase with age ($p < 0.0001$), and also with radiation dose ($p = 0.0060$). There was no difference in the percentage of CD45RO-positive memory cells between males and females (data not shown). As for memory T-cell subsets (M1, M2, and M3), the percentage of each subset in the CD4 T-cell population appeared to significantly increase with age ($p < 0.0001$); but again, these percentages did not differ between males and females (data not shown). The percentages of M2 ($p = 0.0001$) and M3 ($p = 0.0096$) subsets were found to significantly increase with radiation dose.

Table II. Crude means of the percentages of memory subsets in the CD4 T-cell population by age category.

	Age category		
	60–69 yrs	70–79 yrs	≥80 yrs
Subset	Mean 65.5 yrs	75.7 yrs	84.8 yrs
CD45 RO (total memory)	48.8 (1.10)[a]	52.2 (0.85)	58.1 (0.87)
M1 (mature, fully competent)	17.3 (0.62)	18.7 (0.49)	22.4 (0.56)
M2 (immature, poorly competent)	26.1 (0.57)	27.4 (0.43)	29.3 (0.45)
M3 (death prone, anergic)	5.3 (0.14)	6.1 (0.17)	6.4 (0.14)

[a]Standard error in parentheses.

Table III. Crude means of the percentages of memory subsets in the CD4 T-cell population by dose category.

	Radiation dose category			
	<0.005 Gy	0.005–0.5 Gy	0.5–1.0 Gy	1.0–4.0 Gy
Subset	Mean 0.0 Gy	0.20 Gy	0.75 Gy	1.74 Gy
CD45 RO	53.3 (0.93)[a]	53.1 (0.99)	55.0 (1.49)	54.6 (1.13)
M1	19.8 (0.57)	20.1 (0.57)	20.0 (0.88)	19.3 (0.72)
M2	27.4 (0.47)	27.2 (0.51)	29.0 (0.75)	29.0 (0.57)
M3	6.1 (0.18)	5.8 (0.14)	6.0 (0.21)	6.3 (0.20)

[a]Standard error in parentheses.

Table IV. Association of the percentages of memory subsets in the CD4 T-cell population with age or dose (multiple regression analysis)[a].

	% change of subset percentage per unit	
Subset	Age (10 years)[b]	Dose (1 Gy)[c]
CD45RO	10.8	4.3
	(8.0, 13.5)[d]	(1.3, 7.3)
	$p < 0.0001$	$p = 0.0060$
M1	14.6	1.3
	(10.1, 19.1)	(−3.7, 6.2)
	$p < 0.0001$	$p = 0.61$
M2	7.3	5.8
	(4.7, 10.0)	(2.9, 8.7)
	$p < 0.0001$	$p = 0.0001$
M3	10.6	4.9
	(7.2, 13.9)	(1.2, 8.6)
	$p < 0.0001$	$p = 0.0096$

[a]Representative memory subset percentage (95% confidence interval) for non-irradiated males at 70 years of age was calculated to be 15.3 (13.9, 16.7) for M1, 24.8 (23.6, 26.0) for M2, and 5.1 (3.9, 6.4) for M3. Note that there was no significant difference in the percentage of total CD45RO-positive memory cells and that of each memory T-cell subset between males and females. [b]Effects of age were estimated for 10 years. [c]Effects of radiation dose were estimated for 1 Gy. [d]95% confidence interval.

These radiation dose-related changes of memory T-cell subsets observed within the CD4 T-cell population may also involve comparable changes within memory subsets of the CD45RO-positive CD4 T-cell population (Table V). The percentages of M1 and M2 subsets in the memory CD4 T-cell population appeared to significantly increase and decrease with age ($p = 0.0085$ and $p < 0.0001$), respectively. In association with radiation dose, there was a statistically significant decrease in the percentage of M1 subset within the CD45RO-positive memory CD4 T cell population ($p = 0.039$). The ratio of the M1 subset to the combined M2 and M3 subsets also significantly decreased with radiation dose ($p = 0.043$), in contrast to a significant increase in this ratio with age ($p = 0.0030$).

Discussion

Our previous study (Ohara et al. 2002) has clearly shown functional differences among M1, M2, and M3 memory T-cell subsets: Cells in the M1 subset have greater capacity to respond to recall antigens (such as tuberculosis purified protein derivative and tetanus toxoid) and to secrete interferon-γ and IL-4 than cells in either of the other subsets; the M2 subset is comprised of memory-type cells that are less mature than cells of the M1 subset, in terms of not only their memory cell function (i.e., recall antigen reactivity and cytokine-producing ability), but also in terms of their chromosomes' telomere length (longer telomeres); and the M3 subset, in contrast to the M2 subset, consists of cells that are anergic to TCR-mediated stimuli and prone to apoptosis. Therefore, an increase in the proportion of these functionally less competent T-cell subsets (i.e., M2 and M3) may

Table V. Comparable changes of memory subsets within CD45RO-positive memory CD4 T-cell population with age or dose (multiple regression analysis).

	% change of subset percentage per unit	
Subset	Age (10 years)[a]	Dose (1 Gy)[b]
M1	3.5	−3.0
	(0.9, 6.1)[c]	(−5.8, −0.2)
	$p = 0.0085$	$p = 0.039$
M2	−3.2	1.5
	(−4.6, −1.8)	(−0.6, 3.0)
	$p < 0.0001$	$p = 0.059$
M3	−0.2	0.6
	(−3.4, 3.0)	(−2.9, 4.1)
	$p = 0.91$	$p = 0.73$
Ratio [M1/(M2+M3)]	6.1	−4.5
	(2.1, 9.0)	(−8.8, −0.1)
	$p = 0.0030$	$p = 0.043$

[a]Effects of age were estimated for 10 years. [b]Effects of radiation dose were estimated for 1 Gy. [c]95% confidence interval.

not be beneficial to the individual in terms of immunological memory to previously encountered foreign antigens. Such preferential expansion of M2 and M3 subsets may also imply an insufficient maturation of antigen-primed CD4 T cells to the fully memory-competent M1 subset within the individuals' immune system. A hypothesis on memory CD4 T-cell differentiation pathways is depicted in Figure 2. After antigen exposure, naive T cells may undergo repeated cycles of cell division and transformation into the premature memory stage M2 cells. The conversion of M2 cells into the fully functioning mature memory stage M1 cells also requires population doublings following antigen exposure. Replication of M1 cells in response to recall antigens is largely responsible for the maintenance of memory functions. M3 cells, by contrast, are likely to be cells that are approaching senescence, and may arise from fully mature M1 cells that have lost survival signals such as cytokine signalling. We can also suspect that premature M2 cells are directly transformed into death-prone M3 cells. Such putative differentiation pathways may be controlled by interaction of memory T cells with antigen-presenting cells and environmental cytokine conditions. Such circumstances of memory T cells are very important to properly maintain immunological memory. In the CD4 T-cell systems of A-bomb survivors, there are at least two possibilities that the differentiation from M2 to M1 cells may be insufficient, and that cell transit from M2 and M1 subsets to apoptotic-prone M3 populations may be enhanced. Effects of radiation on cellular and molecular mechanisms controlling the memory T-cell differentiation pathways remain to be investigated. Taken together, our results suggest that function and maintenance of helper T-cell memory in the immune system of A-bomb survivors might have been compromised, after A-bomb irradiation.

Our previous study has shown that proliferative responsiveness of memory CD4 T cells to recall antigens can be enhanced by triggering cell-surface CD43 molecules with HSCA-2 mAb in vitro (Kyoizumi et al. 2004). That suggests that CD43 molecules play a part in certain of the cell signalling events involved in memory T-cell activation. Further, it is likely that CD43 and CD28 mAbs act synergistically to stimulate CD4 T-cell response to TCR cross-linking in vitro, indicating the co-stimulatory function of CD43 in TCR-mediated activation processes (Kyoizumi et al. 2004). It has also been suggested in the mouse immune system that the up-regulation of CD43 expression can have a negative effect on activation-induced cell death of T cells (He and Bevan 1999). A recent study has indicated that CD43 molecules induce a signalling cascade that prolongs the duration of TCR signal-mediated cell proliferation and cytokine secretion,

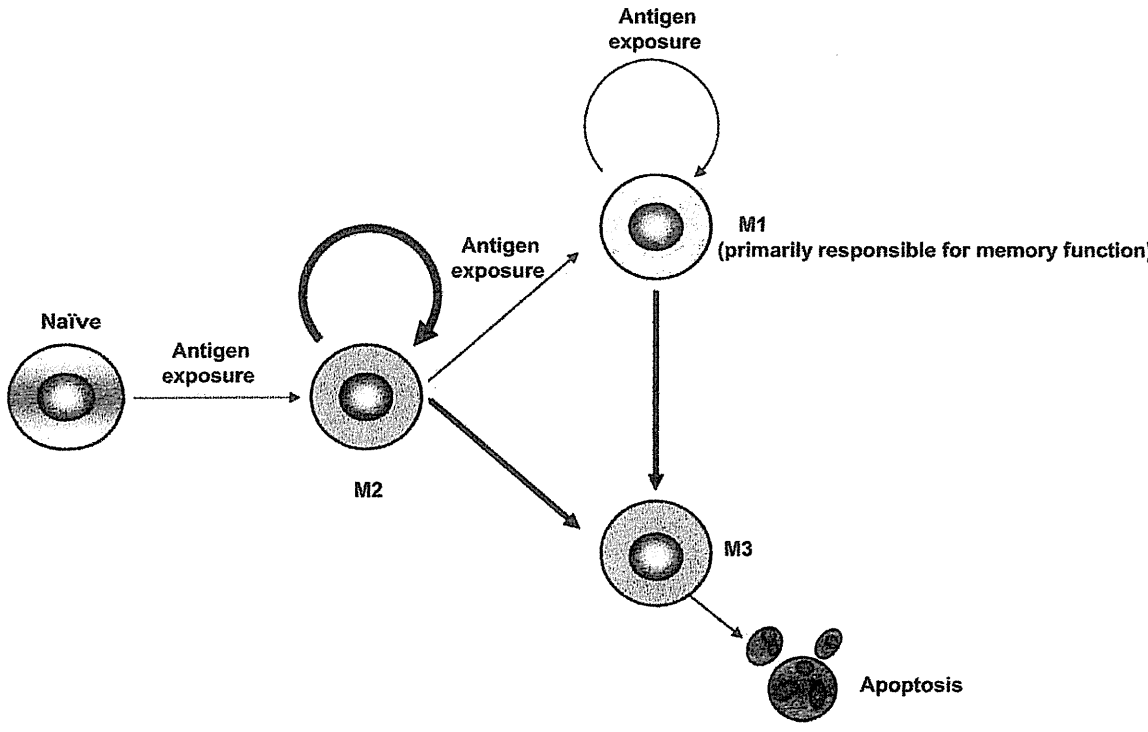


Figure 2. Hypothesised memory CD4 T-cell differentiation pathways for A-bomb survivors. Preferential pathways in the survivors' immune systems are drawn with bold lines.

but that prevents TCR signal-mediated anergy (Fierro et al. 2006). Thus, evidence is accumulating that there are positive effects of CD43-mediated signalling on activation and survival of memory T cells. By contrast, studies that have employed gene disruption techniques have shown that CD43 has either a negative regulatory role (Thurman et al. 1998, Tong et al. 2004), or possibly, plays no significant role in T-cell activation (Carlow et al. 2001). Although the precise mechanism of CD43-dependent regulation of T-cell activation remains to be determined, we have clearly demonstrated that CD43 expression is positively correlated with antigen responsiveness of memory CD4 T cells (Ohara et al. 2002). It is highly likely that the preferential increase in select memory subsets that express lower levels of CD43 (M2 and M3) may be associated with attenuated immune responses to specific pathogens. Levels of immunoglobulin G and A to *Chlamydia pneumoniae* have recently been found to decrease significantly with radiation dose among A-bomb survivors (Hakoda et al. 2006). It would be intriguing to study associations between antigen-specific responses to such ubiquitous pathogens and composition of memory T-cell subsets as defined by the relative level of CD43 expression among A-bomb survivors.

The individual's ability to properly maintain T-cell memory is known to decline with age (Goronzy and Weyand 2005, Weng 2006). This ageing-related immune attenuation is thought to be associated with: (i) The reduction in the size of naïve T-cell pool due to reduced production of new T cells within the involuted thymus, and subsequent, but infrequent entry of antigen-primed cells into the memory T-cell pool, and (ii) divergent antigen recognition repertoire of the memory T-cell pool due to the expansion or shrinkage of functionally incompetent memory T-cell populations (Goronzy and Weyand 2005, Weng 2006). Our previous observations of the immune system of A-bomb survivors are consistent with these typical features that relate to immunological ageing. In this regard, the proportion of naïve CD4 T cells was shown to decrease slightly, but significantly, with radiation dose (Kusunoki et al. 1998, 2002, Yamaoka et al. 2004). Also, the extent to which the TCR repertoire deviated from normal in memory CD4 T cells significantly increased with radiation dose in aged survivors (Kusunoki et al. 2003). An age-dependent increase in the percentage of M1 subset within the memory CD4 T-cell population may reflect the frequent expansion of functional memory T-cell populations in aged individuals. As far as we have examined for several individuals of the present study subjects, clonally expanded populations are largely distributed in M1 subset (Kyoizumi, manuscript in preparation), suggesting that, in aged individuals, only a small population of M1 subset may contribute to recall antigen responses in vivo. The observations in the present study can also be interpreted as an attenuation of helper T-cell memory possibly resulting from radiation-induced perturbation of T-cell homeostasis in A-bomb survivors.

Acknowledgements

The authors thank Dr Thomas M. Seed and Dr Evan B. Douple for careful reading of this manuscript. We are also grateful to Mika Yonezawa and Yoko Takemoto for their assistance with the manuscript preparation. The Radiation Effects Research Foundation (RERF), Hiroshima and Nagasaki, is a private non-profit foundation funded by the Japanese Ministry of Health, Labour and Welfare (MHLW) and the United States Department of Energy (DOE), the latter in part through the National Academy of Sciences. This publication was based on RERF Research Protocol RP 4-02 and supported in part by Grants-in-Aid for Scientific Research from Japan's Ministry of Education, Culture, Sports, Science, and Technology and MHLW.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Armitage G, Berry G, Matthews JNS. 2002. Statistical methods in medical research. Oxford: Blackwell Science.

Carlow DA, Corbel SY, Ziltener HJ. 2001. Absence of CD43 fails to alter T cell development and responsiveness. The Journal of Immunology 166:256–261.

Cullings HM, Fujita S, Funamoto S, Grant EJ, Kerr GD, Preston DL. 2006. Dose estimation for atomic bomb survivor studies: Its evolution and present status Radiation Research 166:219–254.

Dutton RW, Bradley LM, Swain SL. 1998. T cell memory. Annual Review of Immunology 16:201–223.

Eiraku N, Hingorani R, Ijichi S, Machigashira K, Gregersen PK, Monteiro J, Usuku K, Yashiki S, Sonoda S, Hall WW. 1998. Clonal expansion within $CD4^+$ and $CD8^+$ T cell subsets in human T lymphotropic virus type I-infected individuals. The Journal of Immunology 161:6674–6680.

Fierro NA, Pedraza-Alva G, Rosenstein Y. 2006. TCR-dependent cell response is modulated by the timing of CD43 engagement. The Journal of Immunology 176:7346–7353.

Fitzgerald JE, Ricalton NS, Meyer AC, West SG, Kaplan H, Behrendt C, Kotzin BL. 1995. Analysis of clonal $CD8^+$ T cell expansions in normal individuals and patients with rheumatoid arthritis. The Journal of Immunology 154:3538–3547.

Goronzy JJ, Weyand CM. 2005. T cell development and receptor diversity during aging. Current Opinion in Immunology 17:468–475.

Hakoda M, Kasagi F, Kusunoki Y, Matsuura S, Hayashi T, Kyoizumi S, Akahoshi M, Suzuki G, Kodama K, Fujiwara S. 2006. Levels of antibodies to microorganisms implicated in

atherosclerosis and of C-reactive protein among atomic bomb survivors. Radiation Research 166:360–366.

He YW, Bevan MJ. 1999. High level expression of CD43 inhibits T cell receptor/CD3-mediated apoptosis. The Journal of Experimental Medicine 190:1903–1908.

Kodama K, Mabuchi K, Shigematsu I. 1996. A long-term cohort study of the atomic-bomb survivors. Journal of Epidemiology 6(Suppl.):S95–105.

Kusunoki Y, Hirai Y, Hayashi T, Kyoizumi S, Takahashi K, Morishita Y, Kodama Y, Akiyama M. 1993. Frequent occurrence of in vivo clonal expansion of $CD4^-$ $CD8^-$ T cells bearing T cell receptor $\alpha\beta$ chains in adult humans. European Journal of Immunology 23:2735–2739.

Kusunoki Y, Kyoizumi S, Hirai Y, Suzuki T, Nakashima E, Kodama K, Seyama T. 1998. Flow cytometry measurements of subsets of T, B and NK cells in peripheral blood lymphocytes of atomic bomb survivors. Radiation Research 150:227–236.

Kusunoki Y, Yamaoka M, Kasagi F, Hayashi T, Koyama K, Kodama K, MacPhee DG, Kyoizumi S. 2002. T cells of atomic bomb survivors respond poorly to stimulation by *Staphylococcus aureus* toxins in vitro: Does this stem from their peripheral lymphocyte populations having a diminished naive CD4 T-cell content? Radiation Research 158:715–724.

Kusunoki Y, Yamaoka M, Kasagi F, Hayashi T, MacPhee DG, Kyoizumi S. 2003. Long-lasting changes in the T-cell receptor V beta repertoires of CD4 memory T-cell populations in the peripheral blood of radiation-exposed people. British Journal of Haematology 122:975–984.

Kyoizumi S, Kusunoki Y, Hayashi T, Hakoda M, Cologne JB, Nakachi K. 2005. Individual variation of somatic gene mutability in relation to cancer susceptibility: Prospective study on erythrocyte glycophorin A gene mutations of atomic bomb survivors. Cancer Research 65:5462–5469.

Kyoizumi S, Ohara T, Kusunoki Y, Hayashi T, Koyama K, Tsuyama N. 2004. Expression characteristics and stimulatory functions of CD43 in human $CD4^+$ memory T cells: Analysis using a monoclonal antibody to CD43 that has a novel lineage specificity. The Journal of Immunology 172:7246–7253.

Maini MK, Casorati G, Dellabona P, Wack A, Beverley PC. 1999. T-cell clonality in immune responses. Immunology Today 20:262–266.

Musette P, Bequet D, Delarbre C, Gachelin G, Kourilsky P, Dormont D. 1996. Expansion of a recurrent V beta 5.3^+ T-cell population in newly diagnosed and untreated HLA-DR2 multiple sclerosis patients. Proceedings of the National Academy of Sciences of the USA 93:12461–12466.

Nakano M, Kodama Y, Ohtaki K, Itoh M, Awa AA, Cologne J, Kusunoki Y, Nakamura N. 2004. Estimating the number of hematopoietic or lymphoid stem cells giving rise to clonal chromosome aberrations in blood T lymphocytes. Radiation Research. 161:273–281.

Ohara T, Koyama K, Kusunoki Y, Hayashi T, Tsuyama N, Kubo Y, Kyoizumi S. 2002. Memory functions and death proneness in three $CD4^+CD45RO^+$ human T cell subsets. The Journal of Immunology 169:39–48.

Posnett DN, Sinha R, Kabak S, Russo C. 1994. Clonal populations of T cells in normal elderly humans: The T cell equivalent to 'benign monoclonal gammapathy'. The Journal of Experimental Medicine 179:609–618.

Silins SL, Cross SM, Krauer KG, Moss DJ, Schmidt CW, Misko IS. 1998. A functional link for major TCR expansions in healthy adults caused by persistent Epstein-Barr virus infection. The Journal of Clinical Investigation 102:1551–1558.

Thurman EC, Walker J, Jayaraman S, Manjunath N, Ardman B, Green JM. 1998. Regulation of in vitro and in vivo T cell activation by CD43. International Immunology 10:691–701.

Waase I, Kayser C, Carlson PJ, Goronzy JJ, Weyand CM. 1996. Oligoclonal T cell proliferation in patients with rheumatoid arthritis and their unaffected siblings. Arthritis & Rheumatism 39:904–913.

Tong J, Allenspach EJ, Takahashi SM, Mody PD, Park C, Burkhardt JK, Sperling AI. 2004. CD43 regulation of T cell activation is not through steric inhibition of T cell-APC interactions but through an intracellular mechanism. The Journal of Experimental Medicine 199:1277–1283.

Wack A, Cossarizza A, Heltai S, Barbieri D, D'Addato S, Fransceschi C, Dellabona P, Casorati G. 1998. Age-related modifications of the human alphabeta T cell repertoire due to different clonal expansions in the $CD4^+$ and $CD8^+$ subsets. International Immunology 10:1281–1288.

Weng NP. 2006. Aging of the immune system: How much can the adaptive immune system adapt? Immunity 24:495–499.

Yamaoka M, Kusunoki Y, Kasagi F, Hayashi T, Nakachi K, Kyoizumi S. 2004. Decreases in percentages of naïve CD4 and CD8 T cells and increases in percentages of memory CD8 T cell subsets in the peripheral blood lymphocyte populations of A-bomb survivors. Radiation Research 161:290–298.

In: HER2 and Cancer
Editor: Sophie I. Williams et al., pp. 187-200

ISBN: 978-1-61122-650-8

Chapter IX

Topoisomerase II-Alpha Index Predicts the Efficacy of Anthracycline-Based Chemotherapy for Breast Cancers

***Kiyomi Taniyama*[*1,2], *Nao Morii*[3,4], *Kazuya Kuraoka*[2], *Akihisa Saito*[2], *Toshinao Nishimura*[2], *Junichi Sakane*[2], *Mieko Harada*[2], *Miho Tanaka*[2], *Hiroyo Takahashi*[3,4], *Kazuaki Miyamoto*[3] and *Hironori Kato*[3]**

[1]Institute for Clinical Research, Departments of [2]Diagnostic Pathology, [3]Breast Surgery and [4]Surgery, National Hospital Organization, Kure Medical Center and Chugoku Cancer Center, Kure, Japan

Abstract

DNA topoisomerase II-alpha (TOPOIIα) has been reported that its gene copy number or protein expression may be predictive of Anthracycline-based chemotherapy or patient's prognosis in breast cancers. Our data indicated the breast cancers with TOPOIIα index ≧ 25 % regressed more effectively than those with TOPOIIα index < 10 % on histology by Anthracycline-based chemotherapy. TOPOIIα index of TOPOIIα gene-deleted tumors did not differ from that of TOPOIIα gene-amplificated or normal tumors. TOPOIIα index, not its gene amplification, is a useful marker of Anthracycline-based chemotherapy in breast cancers.

Keywords: DNA Topoisomerase IIα, Index, Anthracycline, Effect prediction, Ki67

*Corresponding to Kiyomi Taniyama, M.D., Pd.D. Director, Institute for Clinical Research, National Hospital Organization Kure Medical Center and Chugoku Cancer Center, 3-1 Aoyama-cho, Kure 737-0023, Japan. Tel; +81-823-22-3111, Fax: +81-823-22-3273, E-mail: taniyamak@kure-nh.go.jp.

DNA Topoisomerase IIα

DNA topoisomerase is the generic name for an enzyme that severs and reconnects one or both strands of a double-stranded DNA. The enzyme that severs only one of the two strands of a double-stranded DNA is classified as type I, while that which severs both strands is classified as type II. Type II enzyme forms a severing complex by binding to a double-stranded DNA after forming a homodimer. It becomes stable in the presence of Mg^{++} and ATP [1, 2]. Type II enzyme exists in the nucleus and can be classified into α and β, which are expressed from a different gene. Although on the N-terminal side 3/4 of TOPOIIβ is highly homologous with TOPOIIα, it has a different C-terminal domain, and the activity control mechanism exists in this domain.

DNA topoisomerase IIα (TOPOIIα) gene (molecular weight: 170 kDa) is located in the domain 17q21–22, which is near the human epidermal growth factor receptor type 2 (HER2/neu) gene (17q12–21; molecular weight: 185 kDa).

TOPOIIα Index

TOPOIIα protein is a cell proliferation-related antigen, which expresses in the S and G2/M phases during the normal cell cycle. TOPOIIα protein increases by a factor of two to three times in the G2/M phase. Particularly, it expresses most in highly proliferative cells. TOPOIIβ, which has a molecular weight of 180 kDa, constantly exists in any cell and at any phase of the cell cycle; however, its correlation with the therapy-related secondary carcinogenesis has been reported [2, 3]. Although the TOPOIIα index relates to the Caspase-3 index ($p < 0.05$) and the Ki67 index ($p < 0.01$), it is not related to the PCNA index (Figures. 1, 2) [4]. The Ki67 nuclear antigen is not expressed at the silent period (G0); however, it is expressed throughout the cell cycle (i.e., G1, S, G2, and M phases). Ki67 serves as a proliferation marker and a tumor prognostic factor. Although PCNA is also a proliferation-related antigen, its synthesis level begins to increase inside the nucleus at the end of the G1 phase just before the start of DNA synthesis, it maximizes in the S phase and decreases in the G2/M phase. Thus, the expression index of the TOPOIIα protein, which expresses in the S and G2/M phases, only relates to the Ki67 index and not to the PCNA index, even though they are the same cell proliferation-related antigens. Although it is not totally clear why the protein index of Caspase-3, which is a protease that works in the final stage of apoptosis, loosely relates to that of TOPOIIα, one must pay careful attention when evaluating apoptosis for the evaluation of the therapeutic effects of Anthracycline-based chemotherapy, which induces apoptosis from TOPO II inhibition [5–8]. Moreover, the TOPOIIα index not only indicates the proliferation activity but may also relate to the quantitative changes of cancer cells [8, 9].

Ki67 Index

TOPOIIα and Ki67 are often compared as prediction makers for the therapeutic effects of Anthracycline-based chemotherapy. However, because the methods of positive/negative

classification used in the reports vary to a small extent, it is necessary to be careful when comparing the positive rates or evaluating the significance of the measurements. According to Nakopoulou et al., 25% of the invasive breast cancer subjects experienced the TOPOIIα protein expression in more than 10% of the tumor cells.

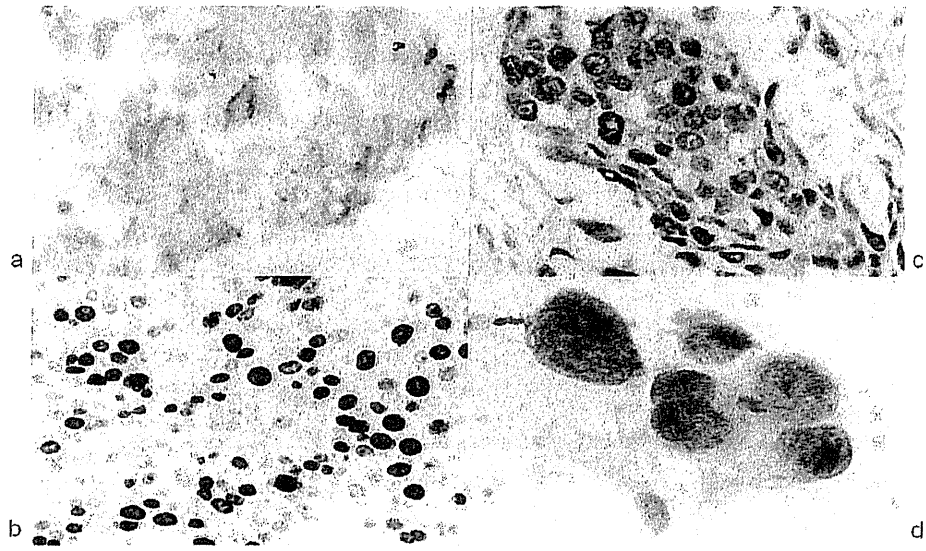


Figure 1. Immunochistochemical findings and cytological atypia of breast cancer cells. (a: Caspase 3 index- 44.8%, b: Ki67 index-18.8%, c: PCNA index-88.7%, d: nuclar atypia grade 3 by Robinson's classification).

The expression of TOPOIIα protein had correlations with nuclear atypia, the Ki67 index, and the p53 index of the tumor cells, as well as hormone receptor non-expression and HER2 protein overexpression in the tumor cells [10]. We investigated the correlation of the positive rate using the 203 infiltrating duct carcinomas as subjects, and reported that the TOPOIIα index had correlations with nuclear atypia (Robinson grade) [11] as well as with the Ki67 index, Caspase-3 index, hormone receptor non-expression, and HER2 gene amplification (Table 1) [4]. However, according to the study conducted by Petit et al., the following facts have been discovered: Of all the factors (nuclear atypia, hormone receptor, Ki67 index, HER2 protein expression, TOPOIIα protein expression, HER2 gene amplification, and TOPOIIα gene amplification), hormone receptor non-expression and the Ki67 index of 20% or greater are effective for the clinical CR (complete response) prediction for neoadjuvant chemotherapy or primary systemic therapy for breast cancer using an anthracycline; and while nuclear atypia has a correlation with the pathological CR prediction, neither protein expressions nor gene amplifications of HER2 and TOPOIIα had any correlation with the therapeutic effect [12]. Meanwhile, Tinari et al. have reported that in the breast cancer cases treated with neoadjuvant chemotherapy or primary systemic therapy using an anthracycline, while HER2 protein and TOPOIIα protein expressions had correlations with the therapeutic effect, neither nuclear atypia nor the Ki67 index had any correlation with the therapeutic effect [13].

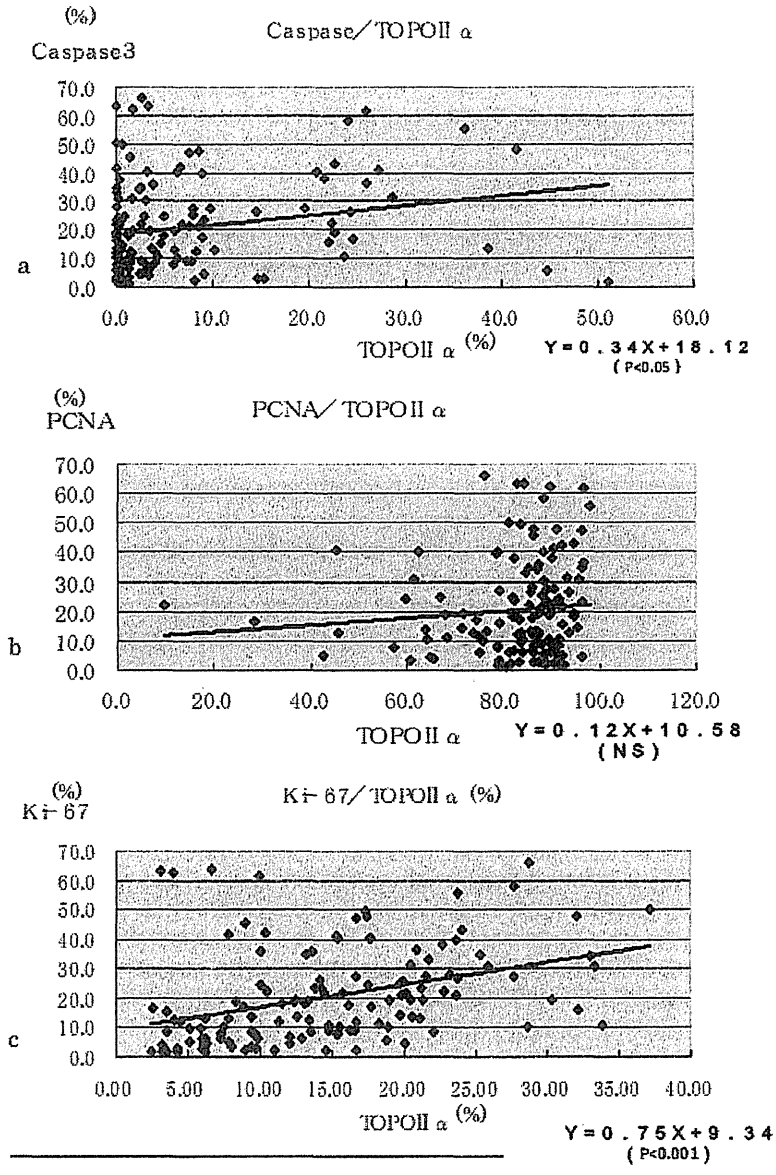


Figure 2. Relations of TOPOIIα index to Caspase 3 index (A), PCNA index (B), and Ki67 index (C), with statistical significance in A and C.

TOPOIIα and HER2 Genes

HER2 genes are the cancer genes that amplify most frequently in breast cancer. One must also be careful when interpreting the results of gene amplification evaluation on these genes because the standards used for evaluation in the previous reports vary. The HER2 gene amplification measured through the fluorescence in situ hybridization (FISH) method can be indicated in the form of signal comparison with CEP17 (Chromosome 17 centromere) (Figure 3). It has been reported that the frequency of occurrence of the signal rate of ≥2.0 is 18%–

29.4% [14–17]. Although monoclonal antibody trastuzumab (Herceptin) is effective in only 23%–26% of the breast cancers having overexpressed HER2 proteins during the simple substance treatment against HER2 proteins, when combined with other anticancer agents the effect will increase up to 50% [17].

Table 1. Relationships among clinicopathological characteristics, topoisomerase II alpha, HER2, Ki67, Caspase 3, and hormon receptors in 203 invasive ductal breast carcinomas of females

Clinico-	Numb er		Tum or	Imunochistochemistry						FISH	
pathologic al characteris tics	of cases	Age	size cm	ER sco re 0—3	Pg R sco re 0—3	Herc ep score 0—3	Ki67(%) %	TOPOII(%) %	Caspase3 (%) %	HER2/C EP index	TOPOIIα/ CEP index
All	203	60, 2	2,3	1,7	1,5	1,0	19,0	18,5	6,3	2,9	1,5
Tumor size											
pT1	112	60, 7	1,4a	1,8	1,5	0,9	18,4	18,1	5,6	2,8	1,4
pT2	79	58, 9	2,9	1,7	1,5	1,0	20,0	19,2	7,1	2,9	1,6
pT3,4	12	63, 3	6,7	1,8	1,3	1,4	17,9	17,7	7,6	4,3	1,7
Pathologic al stage											
I	76	61, 0	1.3a	1,8	1,5	0,8	17,5	16,8	5,9	2,5	1,5
IIA	68	60, 3	2,3	1,8	1,4	1,2	18,9	20,0	5,8	3,4	1,6
IIB	44	58, 9	3,0	1,7	1,7	0,9	20,3	19,3	7,4	2,7	1,4
III, IV	12	60, 3	6,1	1,7	1,1	1,5	18,1	19,4	8,7	4,3	1,7
LN meta (pT1&2)											
absent	110	60, 7	1.8a	1,8	1,5	0,9	17,9	17,4	6,2	2,8	1,5
present	78	59, 2	2,3	1,7	1,5	1,1	19,8	20,3	6,3	3,0	1,5
Robinson grade											
1	92	62. 5b	2,1	2.1 a	1.8 a	0.8a	14.5b	14.9a	3.4c	1.9b	1,4
2	82	59, 0	2,4	1,6	1,4	1,1	20,8	20,5	7,7	3,3	1,6
3	24	54, 2	2,5	0,7	0,6	1,6	30	30,6	12,6	6,1	1,7

Positive cells at ER, PgR score: none, 0; <10%, 1; 10—50%, 2; ≥50%, 3.
a, P<0.001 ; b, P<0.01 ; c, P<0.05.

Jarvinen et al. conducted a study deeming the TOPOIIα / CEP17 signal rate of ≥1.5 as amplification and that of ≤0.7 as deletion, and reported that the TOPOIIα genes were either amplified or deleted in 90% of the breast cancers, in which HER2 genes had been amplified [8, 9]. According to the report by Coon et al. that deemed the signal rate of ≥2.5 as amplification, 23% of the invasive breast cancers showed HER2 gene amplification. Of these

23% tumors, TOPOIIα gene amplification was evident in 67.7%. However, no deletion was observed in either the HER2 genes or TOPOIIα genes [18]. Knoop et al. reported that when deeming the signal rate of ≥2.0 as amplification and that of <0.8 as deletion, the HER2 genes amplified in 29.4% of the breast cancers. In 32.5% of such tumors, the TOPOIIα genes also amplified. Moreover, deletion was observed in 23.6% of the HER2 gene-amplified breast cancers.

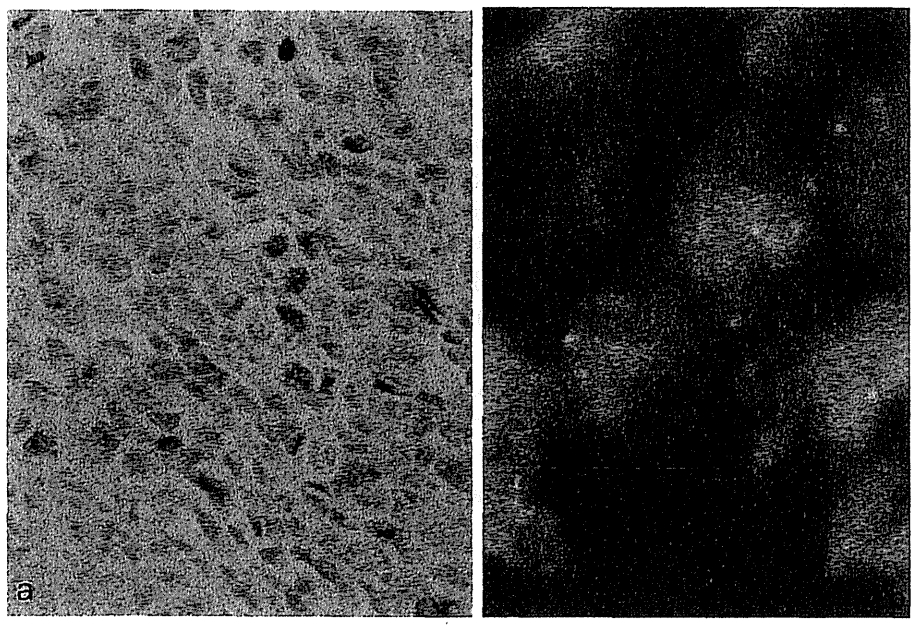


Figure 3. TOPOIIα index of 16.3% (a) and aneuploidy of TOPOIIα gene (b: TOPOIIα / CEP17 = 1.14). Index was evaluated under 40× objective with respect to the positive cell ratio calculated based on the selected five visual fields that are most frequently stained.

In our study, when deeming the signal rate of ≥2.0 as amplification and that of <0.8 as deletion, HER2 gene amplification was observed in 26.6% of the infiltrating duct carcinomas. Of these tumors, TOPOIIα gene amplification and deletion were observed in 25.9% and 11.1% of the tumors, respectively. In addition, when deeming the signal rate of ≥1.5 as amplification, the HER2 gene amplification was observed in 39.9% of the tumors. Of these tumors, TOPOIIα gene amplification and deletion were observed in 51.9% and 8.6% of the tumors, respectively. Moreover, when the HER2 genes were normal (signal rate ranging from 0.8 to 1.5), the TOPOIIα gene amplification was observed in 1.7% at the signal rate of ≥2.0 and in 10.1% at the signal rate of ≥1.5, while deletion was observed in 0.8% [4].

At present, in Japan, the classification of gene amplification is being unified into the HER2 Gene Amplification Standards published by the American Society of Clinical Oncology (ASCO) in 2007. According to these standards, the signal rate of ≥2.2 is deemed as amplification, ranging from 0.8 to 1.8 is deemed as normal, and that of <0.8 is deemed as deletion. The signal rate ranging from 1.8 to 2.2 is classified under the new concept of "equivocal" (borderline region) [15]. Table 1 shows the classification of the results of our study previously described based on these standards. Compared to the data having the signal rate of ≥2.0, the amplification positive rate decreased slightly.

Topoisomerase Inhibition

There are three types of topoisomerase inhibitors: type I inhibitor, type II inhibitor, and dual inhibitor (which inhibits both types). There are two classifications of type II inhibitors: One inhibits the reconnection of the severed double-stranded DNA caused by TOPO II, in doing so it stabilizes the severing complex; and the other inhibits DNA synthesis through the intercalation (bonding) of the double-stranded DNA. Those that display both actions are called intercalators, and those that only show the TOPO II inhibitor action are called non-intercalators [1]. TOPOII inhibitors include anthracyclines (doxorubicin, epirubicin, mitoxantrone, amsacrine, and actinomycin D) as intercalators and epipodophyllotoxins (etoposide and teniposide) as non-intercalators [19].

Anthracyclines, which are intercalators, serve as substrates of P-glycoprotein, and therefore, transfer into cells through passive diffusion [1], where they cause DNA damage due to the TOPO II inhibition and thereby induce apoptosis. They are secreted by P-glycoprotein existing in the brain-vascular barrier. P-glycoprotein high expression, TOPO II protein low expression, and gene mutation are all related to the resistance against the Anthracycline-based chemotherapy.

In addition, because the cardiotoxicity of anthracyclines is irreversible [20], if anthracyclines are used in combination with trastuzumab, cardiac disturbance occurs with high frequency [21]. When administering an anthracyclines, the left ventricular ejection function should be measured using echography and scintigraphy on a regular basis. If the ejection fraction deteriorates, it is important to immediately stop administering the anthracyclines [22].

TopoIIα Genes, Protein Expression, and TopoIIα Inhibitor Sensitivity

TOPOIIα is a molecular target of TOPO II inhibitors, which inhibit the function of TOPOIIα proteins. Thus, the sensitivity of TOPOIIα inhibitors depends on the level of TOPOIIα protein expression of the cancer cells. In other words, a tumor having a low TOPOIIα protein concentration has lower TOPOIIα inhibitor sensitivity than a tumor having a higher TOPOIIα protein concentration. Moreover, in the cancer cells having a high concentration TOPOIIα protein level, hormone receptor non-expression, HER2 protein overexpression, p53 genetic abnormality, DNA aneuploidy, and poor differentiation are observed [9]. Meanwhile, with regard to the TOPOIIα genes, Knoop et al. reported that both TOPOIIα gene amplification and deletion were effective markers for the prediction of anticancer agents, including epirubicin [14]. Epirubicin is one of anthracyclines in which the cardiotoxicity has been reduced [1]. Knoop et al. investigated the HER2 genes and TOPOIIα genes of breast cancers that had been surgically removed prior to the implementation of the CMF (cyclophosphamide, methotrexate, fluorouracil) and CEF (cyclophosphamide, epirubicin, fluorouracil) treatments, using the FISH method. The researchers then investigated the HER2 protein expression immunohistochemically. As a result, it was found that although the state of the HER2 genes had no correlation with the therapeutic effect, the abnormality of

the TOPOIIα genes (i.e., amplification and deletion) correlated with the patient's disease-free survival and the overall increase in the survival rate.

With regard to the TOPOIIα gene deletion, Jarvinen et al. have reported that under the condition of HER2 gene amplification, it occurs with frequency similar to that of amplification (amplification ≥1.5, deletion ≤0.7), and that the TOPO II inhibitor sensitivity decreases [8]. In our study, when deeming the signal rate of ≥1.5 as amplification, HER2 gene amplification was observed in 39.9% of all the infiltrating duct carcinomas. While TOPOIIα gene amplification occurred in 51.9% of the 39.9% infiltrating duct carcinomas, TOPOIIα gene deletion occurred in only 8.6% [4]. Park et al. also investigated breast cancers, deeming the signal rate of ≥1.5 as amplification and that of ≤0.75 as deletion. As a result, HER2 gene amplification was observed in 8.5% of the tumors. Of the 8.5% tumors, TOPOIIα gene amplification occurred in 18 tumors (75%) and deletion occurred in 25% of the tumors. Based on these findings, they reported that TOPOIIα gene deletion does not occur as frequently as amplification [23]. With regard to HER2 gene amplification, the signal rate of ≥2.0 is usually deemed as amplification in the conventional evaluation method. However, because it has been proposed that having the signal rate of ≥2.2 or more than six copies should be deemed as amplification from 2007 [15], it is necessary to organize the frequency of TOPOIIα genetic abnormalities according to the new classification.

Table 2. HER2 gene and TOPOIIα gene in 203 breast cancers (ASCO 2007)

HER2/CEP			TOPOIIα/CEP		
	Amplification ≥2.2	Equivocal 1.8-2.2	Normal 0.8 - 1.8	Deletion <0.8	All
Amplification ≥2.2	12 (23.5%)	4 (7.8%)	29 (56.9%)	6 (11.8%)	51(25.1%)
Equivocal 1.8-2.2	1 (6.7%)	3 (20.0%)	10 (66.7%)	1 (6.7%)	15 (7.4%)
Normal 0.8 - 1.8	3 (2.2%)	6 (4.5%)	124 (92.5%)	1 (0.7%)	134 (66.6%)
Deletion <0.8	0	0	1 (33.3%)	2 (66.7%)	3 (1.5%)
All	16 (7.9%)	13 (6.4%)	164 (80.8%)	10 (4.9%)	203

Table 3. Relationships between HER2 gene and TOPOIIα gene status

HER2/CEP			TOPOIIα/CEP		
	Amplification ≥2.0	Gain 1.5-2.0	Normal 0.8 - 1.5	Deletion <0.8	All
Amplification ≥2.0	14 (25.9%)	13 (24.1%)	21 (38.9%)	6 (11.1%)	54 (26.6%)
Gain 1.5-2.0	5 (18.5%)	10 (37.0%)	11 (40.7%)	1 (7.4%)	27 (13.3%)
Normal 0.8 - 1.5	2 (1.7%)	10 (8.4%)	106 (89.1%)	1 (0.8%)	119 (58.6%)
Deletion <0.8	0	0	1 (33.3%)	2 (66.7%)	3 (1.5%)
All	21 (10.3%)	33 (16.3%)	139 (68.5%)	10 (4.9%)	203

In our study, when deeming the signal rate of ≥2.0 as amplification, TOPOIIα genetic abnormalities (i.e., amplification and deletion) occurred in 37.0% of the HER2-amplified breast cancers (Table 3). Moreover, when deeming the signal rate of ≥2.2 as amplification, the same phenomena occurred in 35.3% of the same cancers (Table 2). In addition, although it had been shown in previous reports that TOPOIIα genetic abnormalities could be observed only in HER2-amplified breast cancer [24], it was subsequently reported that such genetic abnormalities can also be observed in HER2 non-amplified tumors. Knoop et al. has observed TOPOIIα genetic abnormalities in 56.9% of the HER2-amplified tumors (with the signal rate of ≥2.0) and in 7.6% of the HER2 non-amplified tumors [14]. Park et al. also found TOPOIIα genetic abnormalities in 0.95% of the entire group of HER2 non-amplified tumors [23]. In our study, when deeming the HER2 genetic normality as 0.8–1.8, amplification was observed in 2.2% of the tumors and deletion was observed in 0.7% of the tumors (Table 2).

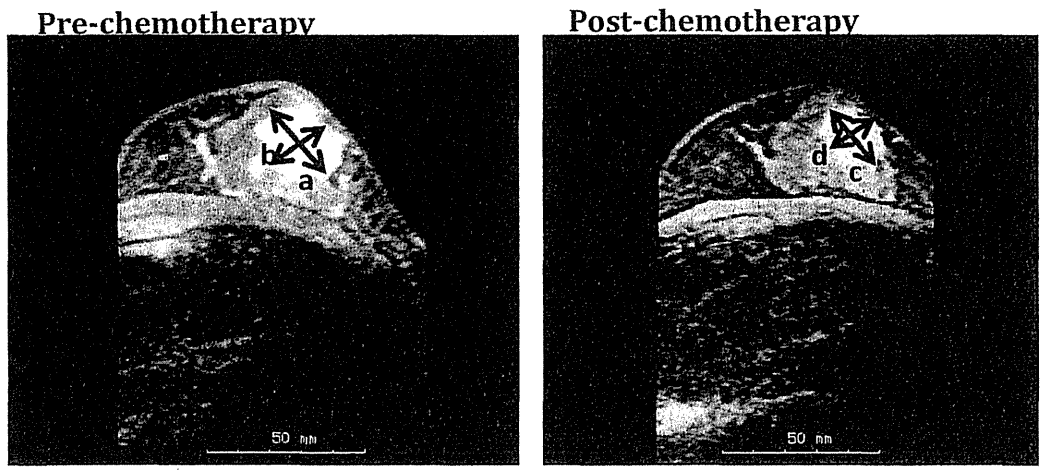
The TOPO II inhibitor sensitivity depends on the level of TOPOIIα protein expression of the cancer cells. Table 4 indicates the results of comparison among the TOPOIIα gene, the TOPOIIα index, the Ki67 index, and the HER2 gene in 172 infiltrating duct carcinomas. There was no difference between the TOPOIIα index of the TOPOIIα gene-deleted tumors and that of other groups. Moreover, there was no difference between the Ki67 index of the TOPOIIα gene-deleted tumor and that of the amplified tumors. Furthermore, the Ki67 index of the TOPOIIα gene amplification cases (signal rate of ≥2.2) significantly increased in comparison to the normal cases (signal rate ranging 0.8–1.8) . We performed the neoadjuvant chemotherapy with anthracyclines for 12 infiltrating ductal carcinomas of the breast and evaluated the tumor reduction rates (Figure 4) after the chemotherapy. As a result, we found a statistically significant ($p = 0.01$) correlation of the tumor reduction rate with the TOPOIIα index, but not with TOPOIIα or HER2 gene amplification (Figure 5). Moreover, among the 28 tumors in which the neoadjuvant chemotherapy with anthracyclines was performed, the correlation between the TOPOIIα index and the tumor regression rates had been evaluated. Consequently, it was observed that the tumor regression rate increased more significantly in the breast cancers in which the TOPOIIα index was evaluated ≥25% than the tumors in which the TOPOIIα index was evaluated <10% (Figure 6). Although these 28 cases included no case of TOPOIIα gene deletion, PR (partial response) with the regression rate of 22% was observed in a single TOPOIIα gene-deletion case (TOPOIIα gene signal rate: 0.69, TOPOIIα protein index: 17.0%, and Ki67 index: 53.5%), on which the clinical image evaluation was conducted after administering the anthracyclines and taxane. These facts suggest the possibility that the TOPOIIα index, immunohistochemically calculated on the tissue obtained prior to the Anthracycline-based chemotherapy, may serve as a prediction factor for the effects of TOPO II inhibitors. In addition, Tinari et al. conducted a study on the breast cancer cases in which neoadjuvant chemotherapy or primary systemic therapy was performed using the anthracyclines. They reported that the HER2 protein expression and TOPOIIα protein expression correlated to the therapeutic effects, and the cases in which the level of TOPOIIα protein expression had increased after the therapy showed significantly low survival rates [13]. Considering this data, when using the TOPO II inhibitor for chemotherapy, it is important to calculate the immunohistochemical TOPOIIα index in the tissues obtained before and after chemotherapy, particularly in terms of prediction for the therapeutic effect of the anthracyclines as well as the prediction of patient prognosis.

Table 4. Comparison of TOPOIIα gene, TOPOIIα Index, Ki67 index and HER2 gene in 172 infiltrating ductal carcinomas of the breast

TOPOIIα	Number	TOPOIIα	Ki67	Number of HER2			
				A	E	N	D
Amplification ≥2.2	15 (8.7%)	19.7%	26.6%	11	1	3	0
Equivocal 1.8-2.2	9 (5.2%)	20.8%	15.9%	2	2	5	0
Normal 0.8-1.8	140 (81.4%)	16.9%	18.1%	19	9	111	1
Deletion <0.8	6 (3.5%)	22.9%	29.7%	4	1	1	0

a, b

A,Amplification; E,Equivocal; N, Normal; D, Deletion
[a]$P = 0.038$(Welch), $P = 0^{b}.022$(student t)



Reduction rate = (a + b) - (c + d) / a + b

Figure 4. Tumor reduction rate calculating the tumor diameter before and after the chemotherapy.

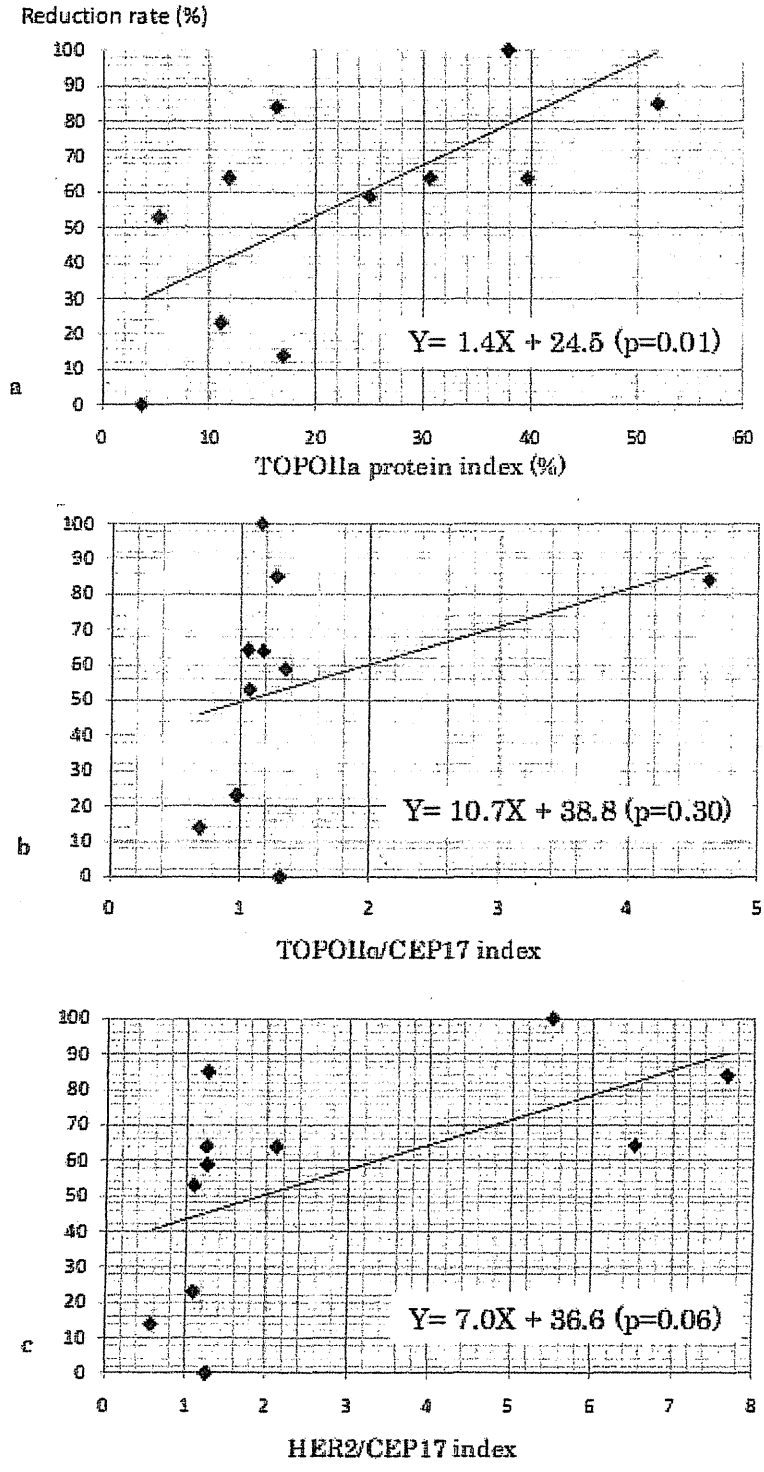


Figure 5. Relations of the tumor reduction rates to the TOPOIIα index (a; p = 0.01), TOPOIIα gene (b; p = 0.30) and HER2 gene (c; p = 0.06).

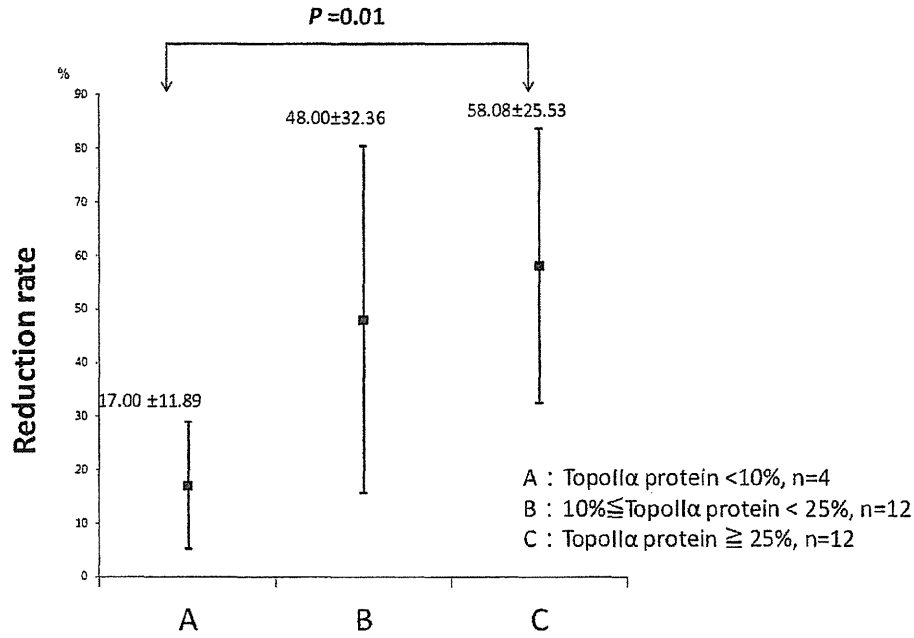


Figure 6. Correlation between the TOPOIIα index and the tumor regression rate.

References

[1] Kato K, et al: Topoisomerase inhibitors. Japanese Clinical Oncology Committee ed.,In; *New Clinical Oncoogy forChemotherapy by Clinical Oncologist*. p274-280, Nankodo, Tokyo, 2008 (in Japanese)

[2] Pommier Y, et al: Topoisomerase II Inhibitors: The Epipodophyllotoxins, Acridines, and Ellipticines. Cancer Chemotherapy and Biotherapy Principles and Practice. (Chabner BA, Longo DL, editors.) p451-475, Lippincott Williams and Wilkins. Philadelphia: 2006.

[3] Toyoda E, et al: NK314, a topoisomerase II inhibitor that specifically targets the alpha isoform. *J. Biol. Chem*. 283:23711-23720, 2008 (in Japanese).

[4] Taniyama K., et al: DNA topoisomerase II alpha. Toi M. ed., In: Basic and Clinic of Breast Cancers for Everyone. p377-388, *IYAKU Journal*, Osaka, 2009 (in Japanese).

[5] Okamoto N., et al. Relations among cellular atypia, proliferative activity and TOPOIIα expression in ductal carcinoma of the breast. *Proceeding of the 59th annual meeting of national hospitals in Japan*. 2005:416 (in Japanese).

[6] Gruber BM, et al: Relationship between topoisomerase II-DNA cleavable complexes, apoptosis and cytotoxic activity of anthracyclines in human cervix carcinoma cells. *Anticancer Res*. 25:2193-2198, 2005.

[7] Chang J, et al: Apoptosis and proliferation as predictors of chemotherapy response in patients with breast carcinoma. *Cancer*. 89:2145-2152, 2000.

[8] Jarvinen TA, et al: Amplification and deletion of topoisomerase IIalpha associate with ErbB-2 amplification and affect sensitivity to topoisomerase II inhibitor doxorubicin in breast cancer. *Am. J. Pathol.* 156:839-847, 2000.

[9] Jarvinen TA, et al: HER2/neu and topoisomerase IIalpha--simultaneous drug targets in cancer. *Comb. Chem. High Throughput Screen.* 6:455-470, 2003.

[10] Nakopoulou L, et al: DNA topoisomerase II-alpha immunoreactivity as a marker of tumor aggressiveness in invasive breast cancer. *Pathobiology.* 68:137-143, 2000.

[11] Robinson, I. A.et al: Typing and grading breast carcinoma on fine-needle aspiration: is this clinically useful information? *Diagn Cytopathol.* 13: 260-265, 1995.

[12] Petit T, et al: Comparative value of tumor grade, hormonal receptors, Ki-67, HER2 and topoisomerase II alpha status as predictive markers in breast cancer patients treated with neoadjuvant anthracycline-based chemotherapy. *Eur. J. Cancer.* 40:205-211, 2004.

[13] Tinari N, et al: Changes of topoisomerase IIalpha expression in breast tumors after neoadjuvant chemotherapy predicts relapse-free survival. *Clin. Cancer Res.* 12:1501-1506, 2006.

[14] Knoop AS, et al. Retrospective analysis of topoisomerase IIa amplifications and deletions as predictive markers in primary breast cancer patients randomly assigned to cyclophosphamide, methotrexate, and fluorouracil or cyclophosphamide, epirubicin, and fluorouracil: Danish Breast Cancer Cooperative Group. *J. Clin. Oncol.* 23:7483-7490, 2005.

[15] Wolff AC, et al. American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer. *Arch. Pathol. Lab. Med.* 131:18-43, 2007.

[16] Pauletti G, et al: Assessment of methods for tissue-based detection of the HER2/neu alteration in human breast cancer: a direct comparison of fluorescence in situ hybridization and immunohistochemistry. *J. Clin. Oncol.* 18:3651-3664, 2000.

[17] Taniyama K, et al: Tyrosine1248-phosphorylated HER2 expression and HER2 gene amplification in female invasive ductal carcinomas. Breast Cancer. 15:231-240, 2008.

[18] Coon JS, et al: Amplification and overexpression of topoisomerase II_alpha predict response to anthracycline-based therapy in locally advanced breast cancer. *Clin. Cancer Res.* 8:1061-1067, 2002.

[19] Inoue K. Mechanisim of the anticancer drugs. Ito Y. and Toi M., ed., In: Breast disease-State of arts. p335-337, *ISHIYAKU shuppan*, Tokyo, 2004 (in Japanese).

[20] Katsumata N.: trastuzumab. Japanese Clinical Oncology Committee ed.,In; New Clinical Oncology forChemotherapy by Clinical Oncologist. p312-315, Nankodo, Tokyo, 2008 (in Japanese)

[21] Seidman A, et al: Cardiac dysfunction in the trastuzumab clinical trials experience. *J. Clin. Oncol.* 20:1215-1221, 2002.

[22] Okamoto M., et al: Maintenance therapy 1) How to treat the adverse effect of drugs used for chemotherapy. Japanese Clinical Oncology Committee ed.,In; *New Clinical Oncology for Chemotherapy by Clinical Oncologist.* p716-723, Nankodo, Tokyo, 2008 (in Japanese)

[23] Park K, et al: Topoisomerase II-alpha gene deletion is not frequent as its amplification in breast cancer. *Breast Cancer Res. Treat.* 98:337-342, 2006.

[24] Jarvinen TA, et al: HER2/neu and topoisomerase II_alpha in breast cancer. *Breast Cancer Res. Treat.* 78:299-311, 2003.