compared with MAs (Fig. 4A), and these genes are listed in Supplementary Table S3K. Of these 194 genes, 128 were considered for functional analysis using IPA. Highly expressed genes common to DFAT and iPS cells are associated with various cell proliferation-related functions, including "mitosis" (22), "M phase" (14), "cell division process" (38), and "cell cycle progression" (27).

4. Discussion

This is the first study investigating the gene expression profiles of multipotent DFAT cells derived from MAs. Using well-established models of MA dedifferentiation [3–6] and advanced microarray technology, similarly expressed as well as significantly differentially expressed genes in DFAT cells and MAs during dedifferentiation were successfully identified. We then pursued in-depth comprehensive bioinformatics analyses, including *de novo* functional annotation and curation of the generated data within the context of biological processes, to unravel several important biological functions associated with dedifferentiation. Finally, we compared the gene expression profiles of DFAT cells with those of iPS cells.

To characterize gene expression signatures of dedifferentiation, genes differentially expressed during dedifferentiation were assigned to functional categories using IPA. We first examined the genes downregulated during MA dedifferentiation and then showed that the number of genes participating in lipid metabolism decreased significantly during dedifferentiation. These results are consistent with those of our previous report, showing that real-time RT-PCR detected the abundant expression of markers, including *LPL*, *LEP*, *GLUT4* (glucose transporter type 4), and *PPARG*, in MAs; however, these markers were not expressed in DFAT cells [4–6]. Thus, findings from the present study and previous reports clearly demonstrate the occurrence of MA dedifferentiation through a significant decrease in the levels of typical genes expressed in functional cells prior to the acquisition of multipotency.

During MA dedifferentiation, 368 genes were significantly upregulated. A close examination of these genes revealed that the functional categories could be divided into "cell proliferation", "altered cell morphology", and "regulation of differentiation".

A wide variety of cell proliferation genes seem to have been expressed during dedifferentiation, particularly a large number of genes involved in "movement of cell", "migration of cells" and "proliferation of cells" (Fig. 3A, Supplementary Table S3B, C and F). In addition, similar observations were found from a comparison of publicly available microarray data on iPS cells. Functional analysis of highly expressed genes in DFAT and iPS cells revealed a large increase in expression levels of cell proliferation-related genes (Fig. 4). While cell dedifferentiation is commonly associated with re-entry into the cell cycle, its distinguishing feature is withdrawal from a given differentiated state into a stem cell-like state, a process that precedes not only re-entry into the cell cycle but also trans- or redifferentiation [18–22]. Using human iPS cells derived from keratinocytes or BJ fibroblasts, Ruiz et al. demonstrated that induction of cell proliferation increases reprogramming efficiency, whereas cell cycle arrest inhibits successful reprogramming [23]. Our results, which used models of MA dedifferentiation, are consistent with these observations of iPS cells in terms of re-entry into the cell cycle. Although there may be several explanations for the positive effect of cell proliferation on dedifferentiation, our data and the above reports suggest that re-entery into the cell cycle may contribute to dedifferentiation itself.

The large categories of genes upregulated during dedifferentiation are that related to "developmental process of tissue" and "differentiation of cells" (Fig. 3C, Supplementary Table S3D and I). MA dedifferentiation resulted in expression of several tissue-specific genes, suggesting that DFAT cells acquire multipotency during dedifferentiation. This could occur by direct alteration of the expression of tissue-specific genes or impacting of genes responsible for controlling cell differentiation. In addition, these results indicate that DFAT cells have features of the progenitor cell committed to various cell lineages rather than the stem cell state. Moreover, in our previous study, we showed that single cell-derived clonal populations of DFAT cells not only expressed the specific genes of various cell lineages but also were capable of differentiation into multiple lineages [6–11]. Therefore, dedifferentiated cells may not be heterogeneous populations, but individual dedifferentiated cells expressing tissue-specific genes and having multilineage differentiation potential. Interestingly, we demonstrated that during dedifferentiation, MAs upregulated several CD

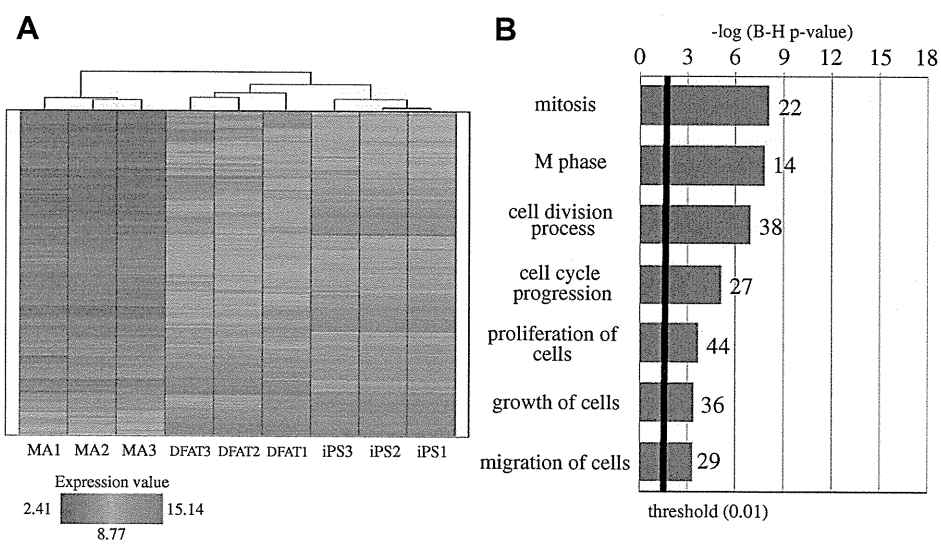

Fig. 4. Comparison of DFAT and iPS cells. (A) Expression of profiled 194 highly expressed genes in DFAT and iPS cells in porcine genome array samples (MAs, DFAT cells, and iPS cells) was compared using hierarchical clustering by Ward's method. (B) Functional classification of highly expressed genes in DFAT and iPS cells. Analyses were performed on 163 genes using IPA. All functional categories demonstrated enhanced statistical representation. Bars represent the proportion of genes involved in each category for which statistical significance and number of genes are indicated.

marker genes not only of MSCs (*CD44*, *CD140a*, *CD140b*, *CD146*, *CD266*, *CD325*, and *CD332*) [24] but also of hematopoietic cells (Fig. 4). From this result, we assumed that DFAT cells might differentiate into several cell types such as hematopoietic cells. The transdifferentiation potential of DFAT cells further confirms the identity of DFAT cells as a multipotent progenitor. Although still controversial, our findings suggest that expression of several tissue-specific genes is one of the transcriptional signatures of dedifferentiated cells and that differentiated mammalian cells could dedifferentiate into the multipotent cell state.

In conclusion, our observations indicate that the transcriptional signatures of DFAT cells derived from MAs are summarized in terms of a significant decrease in functional phenotype-related genes and significant increase in cell proliferation, altered cell morphology, and regulation of differentiation. Furthermore, considering these results, it could be suggested that dedifferentiation in mammals occurs in other somatic cells during culture *in vitro* as well as in MAs. Since components of these processes were detected as being differentially expressed, regulation of these genes could affect new signaling pathways uniquely employed during dedifferentiation. This approach using our experimental procedures will be of use not only for a better understanding of the mechanism of dedifferentiation but also for application in many tissue engineering strategies and cell-based therapies. Therefore, further studies are needed to assess the developmental potency of other differentiated functional cells and to illustrate the molecular basis that establishes and maintains multipotency during dedifferentiation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.03.063.

References

[1] P.R. Johnson, A.L. Goldstein, Cell culture system in obesity research, in: M.R.C. Greenwood (Ed.), Obesity, Churchill Livingstone, Edinburgh, Harlow, 1983, pp. 159–191.
[2] R. Van, The adipocyte precursor cell, in: A. Cryer, R.L.R. Van (Eds.), New Perspectives in Adipose Tissue: Structure Function and Development, Butterworth-Heinemann, London, UK, 1985, pp. 353–382.
[3] K. Yagi, D. Kondo, Y. Okazaki, K. Kano, A novel preadipocyte cell line established from mouse adult mature adipocytes, Biochem. Biophys. Res. Commun. 321 (2004) 967–974.
[4] H. Nobusue, T. Endo, K. Kano, Establishment of a preadipocyte cell line derived from mature adipocytes of GFP transgenic mice and formation of adipose tissue, Cell Tissue Res. 332 (2008) 435–446.
[5] H. Nobusue, K. Kano, Establishment and characteristics of porcine preadipocyte cell lines derived from mature adipocytes, J. Cell Biochem. 109 (2010) 542–552.
[6] T. Matsumoto, K. Kano, D. Kondo, N. Fukuda, Y. Iribe, N. Tanaka, Y. Matsubara, T. Sakuma, A. Satomi, M. Otaki, J. Ryu, H. Mugishima, Mature adipocyte-derived dedifferentiated fat cells exhibit multilineage potential, J. Cell Physiol. 215 (2008) 210–222.
[7] T. Kazama, M. Fujie, T. Endo, K. Kano, Mature adipocyte-derived dedifferentiated fat cells can transdifferentiate into skeletal myocytes in vitro, Biochem. Biophys. Res. Commun. 377 (2008) 780–785.
[8] Y. Oki, S. Watanabe, T. Endo, K. Kano, Mature adipocyte-derived dedifferentiated fat cells can trans-differentiate into osteoblasts in vitro and in vivo only by all-trans retinoic acid, Cell Struct. Funct. 33 (2008) 211–222.
[9] Y. Ohta, M. Takenaga, Y. Tokura, A. Hamaguchi, T. Matsumoto, K. Kano, H. Mugishima, H. Okano, R. Igarashi, Mature adipocyte-derived cells, dedifferentiated fat cells (DFAT) Promoted functional recovery from spinal cord injury-induced motor dysfunction in rats, Cell Transplant. 17 (2008) 877–886.
[10] T. Sakuma, T. Matsumoto, K. Kano, N. Fukuda, D. Obinata, K. Yamaguchi, T. Yoshida, S. Takahashi, H. Mugishima, Mature adipocyte derived, dedifferentiated fat cells can differentiate into smooth muscle-like cells and contribute to bladder tissue regeneration, J. Urol. 182 (2009) 355–365.
[11] M. Jumabay, T. Matsumoto, S. Yokoyama, K. Kano, Y. Kusumi, T. Masuko, M. Mitsumata, S. Saito, A. Hirayama, H. Mugishima, N. Fukuda, Dedifferentiated fat cells convert to cardiomyocyte phenotype and repair infarcted cardiac tissue in rats, J. Mol. Cell Cardiol. 47 (2009) 565–575.
[12] M. Rodbell, Metabolism of isolated fat cells I. Effects of hormones on glucose metabolism and lipolysis, J. Biol. Chem. 239 (1964) 375–380.
[13] R.A. Irizarry, B. Hobbs, F. Collin, Y.D. Beazer-Barclay, K.J. Antonellis, U. Scherf, T.P. Speed, Exploration normalization, and summaries of high density oligonucleotide array probe level data, Biostatistics 4 (2003) 249–264.
[14] R. Breitling, P. Armengaud, A. Amtmann, P. Herzyk, Rank products: a simple yet powerful, new method to detect differentially regulated genes in replicated microarray experiments, FEBS Lett. 573 (2004) 83–92.
[15] T. Ezashi, B.P. Telugu, A.P. Alexenko, S. Sachdev, S. Sinha, R.M. Roberts, Derivation of induced pluripotent stem cells from pig somatic cells, Proc. Natl. Acad. Sci. USA 106 (2009) 10993–10998.
[16] T. Barrett, D.B. Troup, S.E. Wilhite, P. Ledoux, D. Rudnev, C. Evangelista, I.F. Kim, A. Soboleva, M. Tomashevsky, K.A. Marshall, K.H. Phillippy, P.M. Sherman, R.N. Muertter, R. Edgar, NCBI GEO: archive for high-throughput functional genomic data, Nucleic Acids Res. 37 (2009) D885–D890.
[17] H. Zola, B. Swart, I. Nicholson, E. Voss, Leukocyte and Stromal Cell Molecules: The CD Markers, John Wiley and Sons, 2007.
[18] W. Bloom, Cellular differentiation and tissue culture, Physiol. Rev. 17 (1937) 589–617.
[19] E.D. Hay, Electron microscopic observations of muscle de-differentiation in regenerating Amblystoma limbs, Dev. biol. 1 (1959) 555–585.
[20] J. Zhao, N. Morozova, L. Williams, L. Libs, Y. Avivi, G. Grafi, Two phases of chromatin decondensation during dedifferentiation of plant cells: distinction between competence for cell fate switch and a commitment for S phase, J. Biol. Chem. 276 (2001) 22772–22778.
[21] K. Echeverri, E.M. Tanaka, Mechanisms of muscle dedifferentiation during regeneration, Semin Cell Dev. Biol. 13 (2002) 353–360.
[22] S.J. Odelberg, Inducing cellular dedifferentiation: a potential method for enhancing endogenous regeneration in mammals, Semin Cell Dev. Biol. 13 (2002) 335–343.
[23] S. Ruiz, A.D. Panopoulos, A. Herrerias, K.D. Bissig, M. Lutz, W.T. Berggren, I.M. Verma, J.C. Izpisua Belmonte, A high proliferation rate is required for cell reprogramming and maintenance of human embryonic stem cell identity, Curr. Biol. 21 (2011) 45–52.
[24] B. Delorme, J. Ringe, N. Gallay, Y. Le Vern, D. Kerboeuf, C. Jorgensen, P. Rosset, L. Sensebe, P. Layrolle, T. Haupl, P. Charbord, Specific plasma membrane protein phenotype of culture-amplified and native human bone marrow mesenchymal stem cells, Blood 111 (2008) 2631–2635.

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Chapter IX

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

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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

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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.

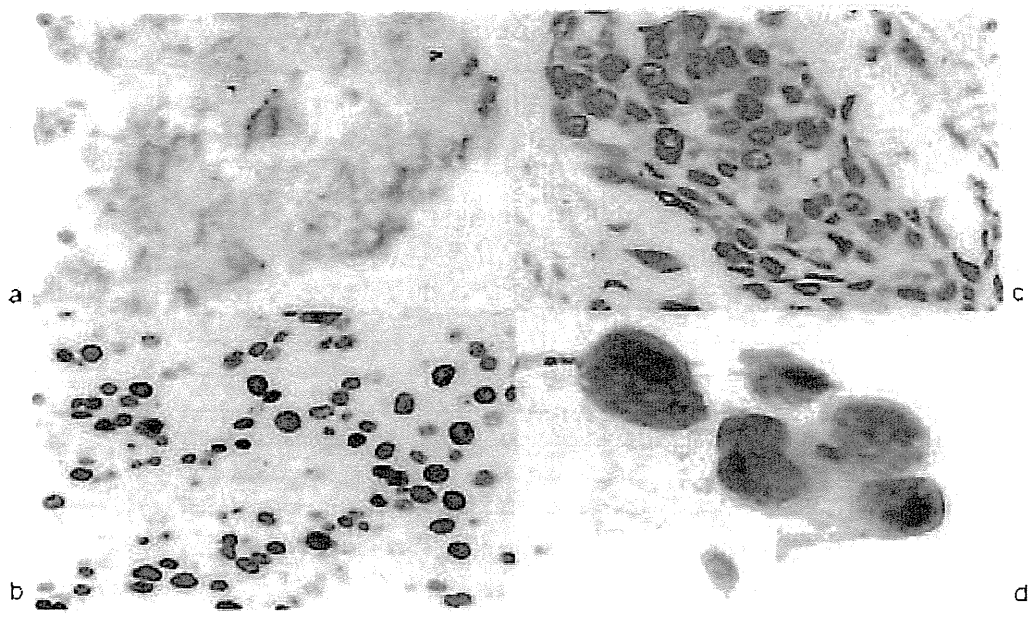


Figure 1. Immunochistochemical findings and cytological atypia of breast cancer cells. (a: Caspase 3 index- 44.8%, b: Ki67 index-18.8%, c: PCNA index-88.7%, d: nuclar atypia grade 3 by Robinson's classification).

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].

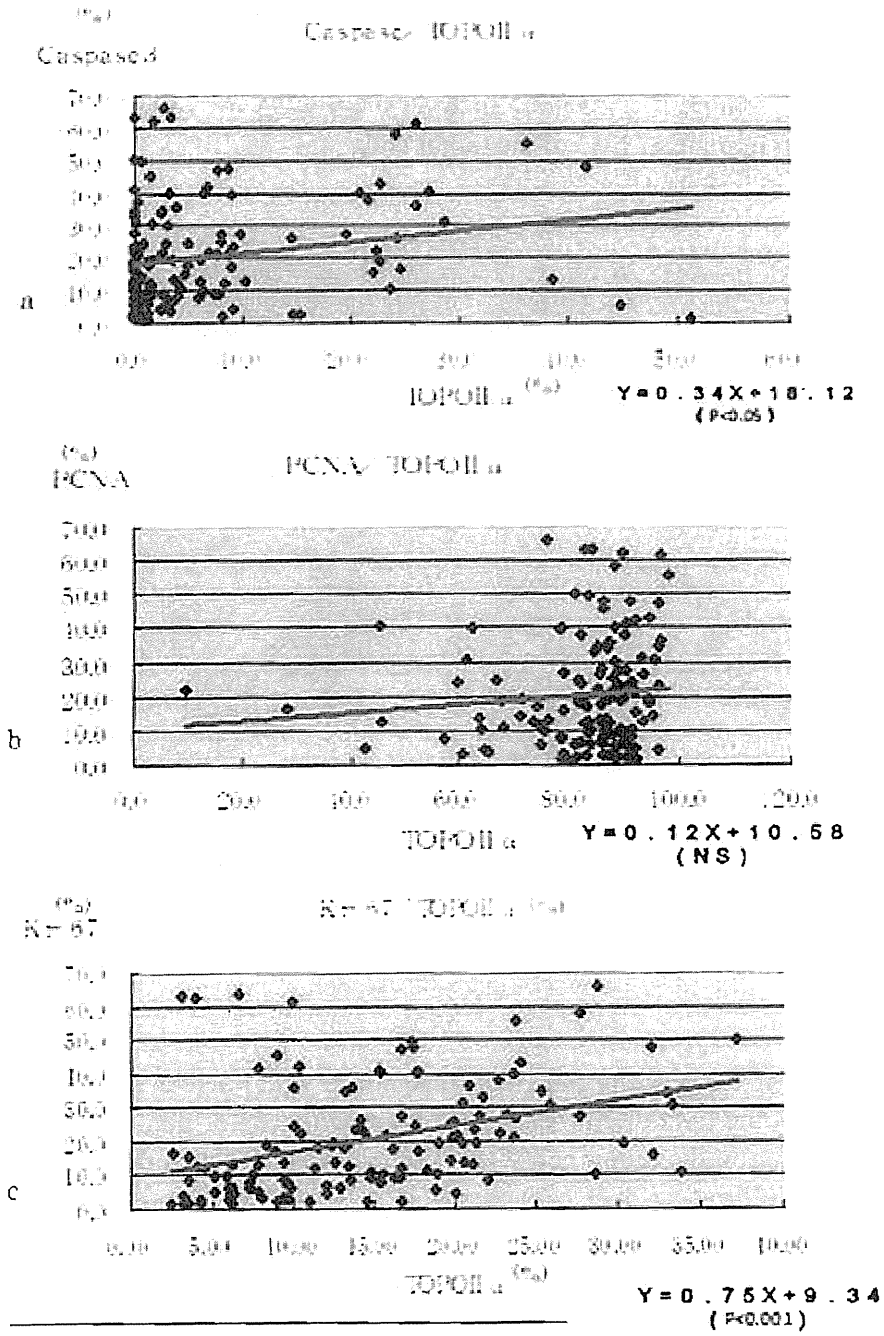


Figure 2. Relations of TOPOIIα index to Caspase 3 index (A), PCNA index (B), and Ki67 index (C), with statistical significance in A and C.

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 scofe 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.

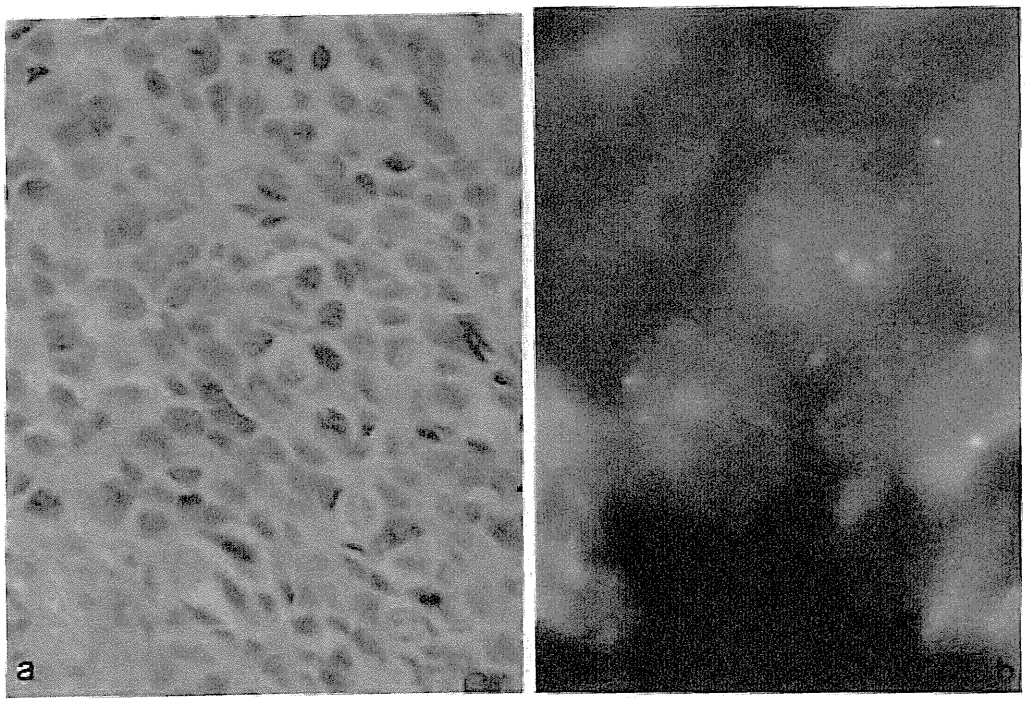


Figure 3. TOPOIIα index of 16.3% (a) and aneuploidy of TOPOIIα gene (b: TOPOIIα / CEP17 = 1.14). Index was evaluated under 40× objective with respect to the positive cell ratio calculated based on the selected five visual fields that are most frequently stained.

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

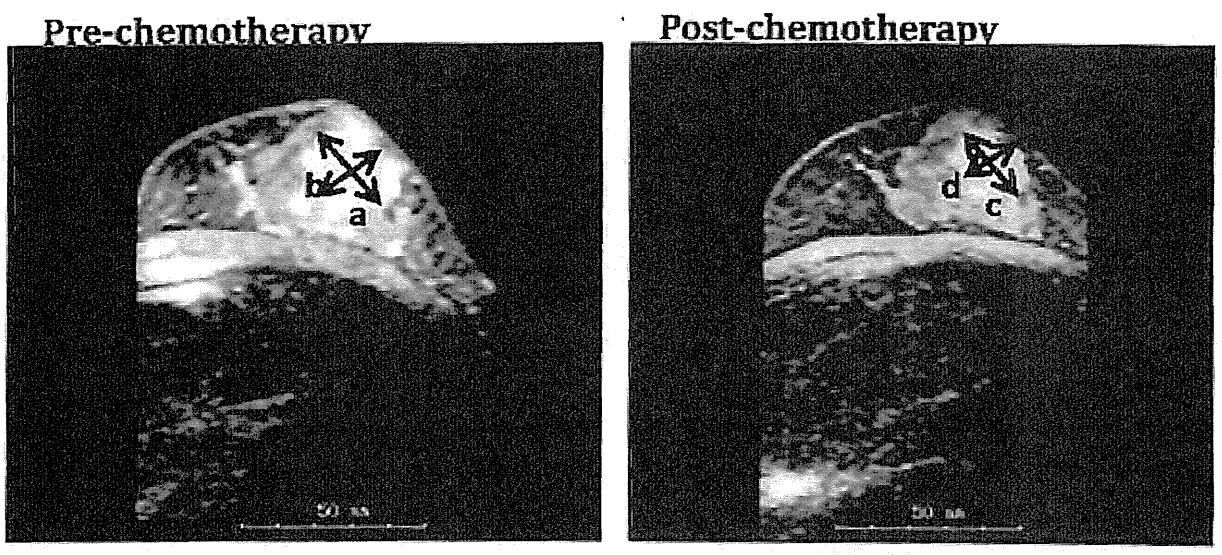
In our study, when deeming the signal rate of ≥2.0 as amplification, TOPOIIα genetic abnormalities (i.e., amplification and deletion) occurred in 37.0% of the HER2-amplified breast cancers (Table 3). Moreover, when deeming the signal rate of ≥2.2 as amplification, the same phenomena occurred in 35.3% 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,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.

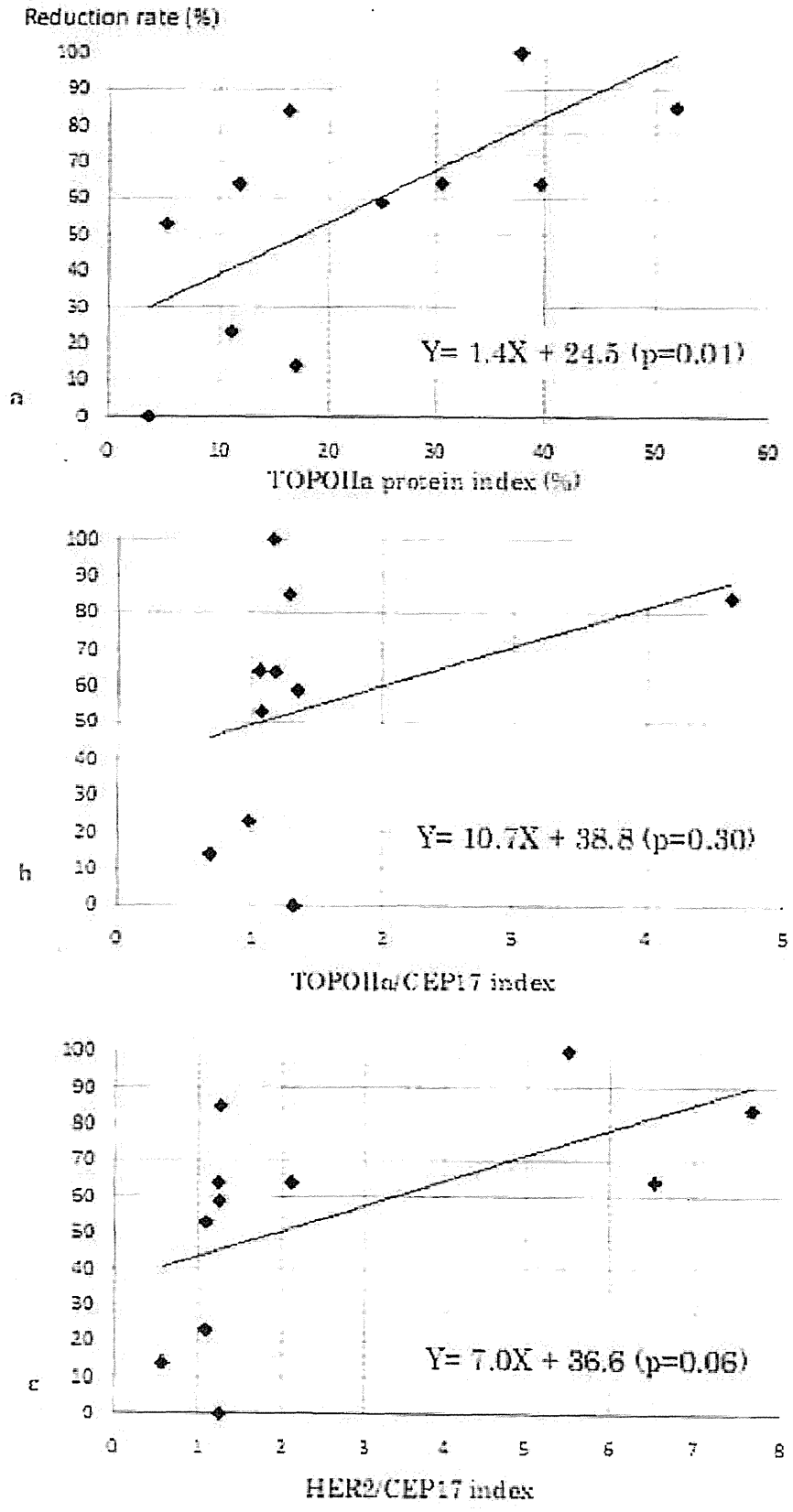


Figure 5. Relations of the tumor reduction rates to the TOPOIIα index (a; p = 0.01), TOPOIIα gene (b; p = 0.30) and HER2 gene (c; p = 0.06).

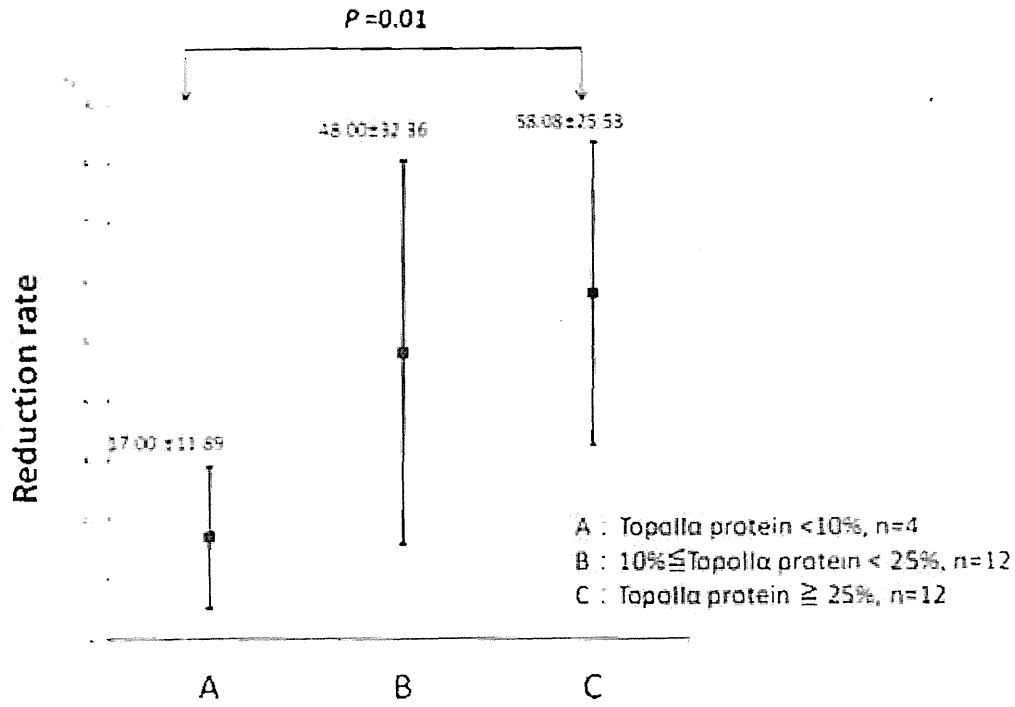


Figure 6. Correlation between the TOPOIIα index and the tumor regression rate.

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.