Identification of genetic origin
in bilateral breast cancer

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Identification of genetic origin
in bilateral breast cancer

Directed by Professor Chung Hyun Cheol

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어느덧 연세교정에서 여섯 번의 봄을 지냈고, 지금 연세에서의 마지막이 될 지도 모를 겨울을 나고 있습니다. 연세라는 이름은 저의 20 대 초반의 즐거움과 슬픔, 모든 추억을 담고 있는 전부였습니다. 그 안에서 늘 큰 사람이 되겠다는 희망으로 살았으며, 누구보다도 큰 꿈으로 시작한 대학원 생활이었습니다. 그리고 꿈을 채 꾸기도 전에 이렇게 또 하나의 학위를 받게 되었습니다.

오늘은 아침부터 보았던 무지개 생각으로 하루 종일 가슴 설레었습니다. 비록, 머리를 감다가 본 목욕탕 타일 바닥을 얇게 적시고 있던 비눗물 사이로 비친 무지개였고, 점심 때 먹던 설렁탕 국물에 비치던 무지개였지만, 저에겐 가슴 벅찬 행복을 느끼기에 충분했습니다. 여유 없이 달려온 2 년 동안 만족보다는 불만으로, 웃음보다는 눈물로 채워져 가는 듯한 저를 강하게 흔들어 줄만큼의 큰 힘을 가진 무지개였습니다. 그렇게 행복한 기분으로 제가 아는 모든 분들께 그 동안 미루어 두었던 감사의 마음을 전하려고 합니다.

먼저, 작은 믿음이지만 힘들 때마다 붙잡고 매달릴 수 있었던 하나님께 감사 드립니다. 늘 제 편에서 항상 웃음을 잃지 말라고, 꿈을 잃지 말라고 가르쳐주신 부모님, 딸 하나에 온 정성을 퍼 부어주신 두분! 고생 많으셨습니다. 그리고 감사 드립니다. 든든함으로 모든걸 표현하는 하나뿐인 우리 오빠, 그 옆의 지현언니 고맙습니다.

2 년 남짓, 그리고 앞으로 더 지속 될 긴 인연, 우리 암전이 연구센터 식구들에게 전하는 마음입니다. 쩌렁쩌렁 울리는 목소리와 그 호탕함으로 연구소를 이끌어 가시는 노재경 소장님. 공부하는 사람의 자세란 무엇인지를 확실히 보여주셨고 특유의 카리스마로 저희 암전이 식구들의 존경을 한 몸에 받고 계시는, 제가 존경하는 정현철 교수님. 몸은 대전에 계시지만, 여기 서울, 암전이 연구센터로 마음의 귀를 기울이고 계시는 인자하신 안성환 교수님. 언니처럼 때론 엄마처럼 다독거려 주시고, 발끝까지 따뜻해지는 미소로 대해주시는 만인의 연인 라선영 교수님. 굳이 말로 표현하지 않아도 전해지는 끈끈한 정으로 학생으로써 연구원으로써의 기본을 가르쳐주시려 애쓰시는 남석우 교수님. 강자 앞에서 더 강하고

약자 앞에서 부드러운 모습이 너무 존경스러운 유내춘 교수님. 우리 암전이 연구센터의 든든한 두 기둥이시며 항상 많은 일을 하시느라 애쓰시는 김태수 선생님, 박규현 선생님. 진심으로 감사드립니다.

연구동 서열 넘버원으로 실험 잘하는 말희언니, 모든 일에 최선을 다하는 모습이 너무 좋은 심웅호 선생님, 우리 후추의 대부이면서 연구동 쥐는 내게 말기라는 면희언니, 늘 함께 공부하자며 이끌어주는 듬직한 큰 오빠 태문쓰, 무슨 얘기를 털어놓아도 말이 새어나갈까 걱정하지 않을 수 있는 우렁이오빠, 맘 착한 수다쟁이 유근이 오빠, 소주 한잔이 생각날 때면 기꺼이 잔을 기울여 주는 푸근한 너털웃음 하진이오빠, 많은 시간을 함께 보내면서 친언니처럼 가까워져 버린 예비 엄마 가비언니, 오랜 친구같이 편안한 현정언니, 조용하고 차분한 주혜언니, 항상 많은 것을 가르쳐 주었고 웃음도 주었던 우리 은송언니, 공부도 열심히 실험도 열심인 순수의 대명사 영, 말수를 부쩍 줄인 모습이 또 색다른 찬희오빠, 자기만의 세계를 사람들에게 서서히 열어 보이고 있는 세원오빠, 동남아 순회공연을 막 마치고 돌아온 우리의 아티스트 재휘오빠, 두 여자 사이에서 갈등했었던(^^) 귀여운 현석오빠, 11월 어느 날 밤의 만행 (오빠들이 시켰어여!!) 에도 가까워질 수 있는 기회를 주었던 년호언니, 한 식구가 된지 얼마 안 돼서 아직은 서먹하지만 곧 본색을 들어내리라 믿어 의심치 않는 정옥언니, 초면의 실례가 있었지만 물질적 정신적으로 많은 도움을 준 웃음쟁이 상철오빠, 연구실 단짝 편도선이 아픈 아이 내 친구 지혜, 늘 즐거운 싹싹한 동생 재희, 가까워지기도 전에 더 좋은 곳으로 떠난다는 동생 진숙이, 리틀주혜 귀여운 막내둥이 경남이, 큰 용기로 다른 길을 찾아 떠났지만 가끔 들려오는 반가운 목소리, 영원한 내 밥과 반찬 효딱이랑 정연이, 새출발을 위해 열심히 노력중인 주영이, 짧은 방학동안 이었지만 갖은 구박에도 끝까지 누나라 불러주는 의외로 성실한 젊은이 태윤이랑 찬주, 그리고 졸업하고 취직해서 얼굴보기 힘들지만 작년 한 해 고맙다는 말을 채 하지 못했던 지현정언니, 연락도 제대로 못하지만 실험실에 밤늦게까지 혼자 남아있을 때면 늘 생각나는 정희철 선생님.
한 페이지를 가득 채워도 모자란 우리 식구들! 너무 고맙습니다!!

다른 학교에서 같은 길을 가고 있는 사랑하는 친구 언영이, 어디서 무엇을 하고 있는지 잘 모르지만 보고 싶은 기원이, 치과의사의 길을 가고

있는 당찬 아람이(틀니 꽁짜로 해주겠다고 한 약속 잊지 않았겠지?), 늦은 나이에 동생들하고 공부하느라 고생이 많은 본과 2 학년 혜련이, 임상연구동에서 이제 대학원 시작으로 캄캄한(ㅎㅎ) 바다와 바람이 아빠 유석, 어느새 아저씨가 되어버린 우리끼리 킹카 장원, 나만큼이나 늘 바쁜 건우, 군대를 간 건지 군인 학교에 간 건지 너무도 잘 살고 있는 문선이, 시커먼 눈썹에서 나오는 카리스마 대기만성형의 균도, 말도 맘도 잘 통하는 친구같은 94 아래층 성재오빠, LG21 세기 선발대로 엮어진 오랜 친구인 늦깎이 본과 1 학년 희택이 오빠, 밥은 잘 먹고 사는지 과학계에 획을 긋고야 말겠다고 유학길에 나선 민정언니, 절 의대 대학원으로 인도했던 보영언니, 학부 때 2 년동안 실험을 참 잘 가르쳐주었던 아라언니, 수민언니.. 생명공학과 97 학번 친구들과 선배님들 교수님 고맙습니다. 제 논문과는 별 상관 없지만, 바쁜 척 하는 저를 늘 잊지 않고 챙겨주고 불러주는 연세 미식축구 이글스 식구들, 저의 연세 6 년을 함께 해준 가족들입니다. 특히 우리 97 학번 동기들 고맙습니다. 그리고 무엇과도 바꿀 수 없는 소중한 친구들.. 미국에 사는 은정이와 전주에 사는 은정, 채연, 혜진, 염지영, 서진, 세라, 은기, 현주, 재선, 혜영, 성국, 나리 등등 제가 아는 분들 고맙다는 말 전합니다.

마지막으로 4 년이 넘는 시간 한결 같은 모습으로 지켜봐 주었던 홍군과, 어렸을 때부터 나의 정신적 지주 동호오빠와 지은언니, 멋쟁이 랩퍼 김진표, 월드컵 4 강의 신화로 생활의 활력을 주었던 우리 태극 전사들에게 감사드립니다.

제 논문은 여러분들 덕분에 마침표를 찍을 수 있었습니다. 부족한 결론으로 마무리 지은 졸업 논문이지만 많은 것을 얻었고 이것 때문에 또 많은 것을 버려야 했습니다. 그리고 이것을 기초로 더 많이 발전할 것입니다.

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Abstract

Identification of genetic origin in bilateral breast cancer

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Bilateral breast cancer (BBC) can be divided into two groups; synchronous and metachronous cancer. In case of both metachronous and synchronous tumors, it remains unclear whether the BBC represents the coincidental occurrence of two independent primary cancers or concurrently identified metastasized tumor from contralateral breast cancer. In later case, two tumors are considered to be of same genetic origin. Understanding about the genetic origin of BBC is very important for prognosis prediction and proper treatment.

In this study, we employed two different technologies in order to assess genetic and epigenetic changes in tumor, which allow us to

determine the genetic characterization of BBC. At first, results from X-chromosome inactivation assay were unable to indicate their genetic origin in bilateral breast cancer. There were some limitations of using X chromosome assay for clinical application in bilateral breast cancer patients. Because of tumor heterogeneity, one marker located on the X chromosome was insufficient for comparing cancer origin in bilateral breast cancer.

Thus, array based CGH pattern analysis was utilized to identify genomic origin in BBC. To evaluate the specificity of the hybridized spot signal on array CGH chip and experimental bias from dye labeling efficiency, we performed homotypic experiment, dye swapping test, and the hybridization with known DNA control spikes. We confirmed that our array-based CGH system specifically recapitulated the genomic changes in target preparation. Different genomic DNA changes were 2%±2.20 in synchronous pairs, whereas the changes were 14.3%±10.6 in metachronous pairs.

In conclusion, our results suggested that bilateral breast cancers which originated from different clones have different chromosomal imbalance patterns. The array based-CGH is considered as a useful tool for direct detection of genome profiles.

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Ⅰ. INTRODUCTION

Tumor metastasis is the major cause of cancer morbidity and mortality. As metastatic cancer cells represent more aggressive behavior and resistant to treatment, metastases cause the major clinical problems in the management of cancer patients. Accordingly, it is very important to define the tumor stage whether the tumor is a primary tumor or metastasized from other primary lesion in order to predict the exact prognosis and provide the optimal treatment to the cancer patient.

One remarkable feature of tumor is the heterogeneity of their constituent cells. Even though there is some debate whether all or a limited number of cells in primary tumor have metastatic potential, it

is clear that individual metastasis originates from a single cell, i.e. one clonal origin and that different metastases can arise from different parent cells. Because metastatic lesions are rarely surgically removed or biopsied, it is hard to evaluate the changes of biomarker during the metastatic progression.

Bilateral breast cancer (BBC) can be divided into two groups; synchronous, in which both tumors occur simultaneously, or metachronous, in which the tumors appear at different points. In case of both metachronous and synchronous tumors, it remains unclear whether the BBC represents the coincidental occurrence of two independent primary cancers or concurrently identified metastasized tumor from contra-lateral breast cancer. In later case, two tumors are considered to be of same genetic origin[1]. The empirical criteria based on the clinical parameter has been used to compare the characterization of two tumors in metachronous and synchronous cancer[2, 3]. The criteria includes; 1) differentiation grade, 2) presence of ductal carcinoma *in situ* (DCIS), and 3) systemic metastasis to another organs.

To identify the characteristics of the various breast cancers clinically, the detection of histological phenotypes such as estrogen

receptor(ER), progesterone receptor(PgR) or c-erbB2 has been used[4]. On the other hand, the differential methylation status in certain X chromosomal genes in females is suggested as a possible molecular approach[5].

The inactivation of one of the two X chromosomes occurs early in development. This process resulted in individuals having cellular mosaics with either the maternal or paternal X chromosome inactivated. Dosage compensation in humans is achieved through the random inactivation of X chromosome. The inactivation of one X-chromosome with concomitant methylation of the 5' end of genes, such as phosphoglycerate kinase(PGK) gene, hypoxanthine phosphoribosyltransferase(HPRT) gene, or human androgen receptor (HUMARA) gene provides a stably inherited genetic marker[6,7,8]. However, there are some limitations of using X chromosome assay for clinical application in bilateral breast cancer patients. Even though the methylation of different X alleles represents absolute evidence of independent tumorigenic processes, the opposite situation with the methylation of the same allele, has little meaning with following reasons[9]. First, because of tumor heterogeneity, one marker dependent on the X chromosome is

insufficient for comparing genetic origin. Second, this assay is applicable only to female. Therefore, a supplementary solution is needed to complement the X chromosome inactivation assay.

The characterization of gene copy number changes and gene expression patterns provide a basis for investigating the pathogenic mechanisms involved in tumor promotion and metastasis. Especially, the information about chromosomal imbalances, such as deletion and amplification, gives us the significant clue to understand malignant behavior of cancer cells. Moreover, individual tumor from the same genetic origin will represent similar patterns in this respect. The DNA microarray technique can be used to monitor many genes at transcription level, simultaneously. Array-based comparative genomic DNA hybridization (CGH) has demonstrated a certain comparative ability in terms of DNA copy-number changes with higher sensitivity and resolution capacity compared to the conventional CGH[10]. This suggests the possibility of using array-based CGH for tumor origin comparison.

In this study, we employed two different technologies in order to assess genetic and epigenetic changes in tumor, which allow us to compare the genetic origin of metachronous and synchronous

bilateral breast cancers. Our results suggest that bilateral breast cancers, which originated from different cell, have different chromosomal imbalance patterns. Array-based CGH is seemed to be an useful tool for the direct genetic profiling.

Ⅱ. MATERIALS AND METHODS

The scheme of the whole study is outlined in Figure1.

1. Tissue specimens.

Eighteen pairs of bilateral breast cancer tissues were obtained as paraffin embedded tissue blocks from biopsy or surgical resection specimens at the Severance Hospital, Yonsei University College of Medicine, Seoul, Korea. Actual experiment sample sizes are showed in Table 1. Pathologist confirmed the diagnosis and tumor areas by hematoxylin and eosin (H&E) stained slide. Tumor samples with high normal cell contents (more than 30% of tissue area) were excluded from the study.

When two breast tumors were detected with the interval of more than one year, we defined it as a metachronous tumor.
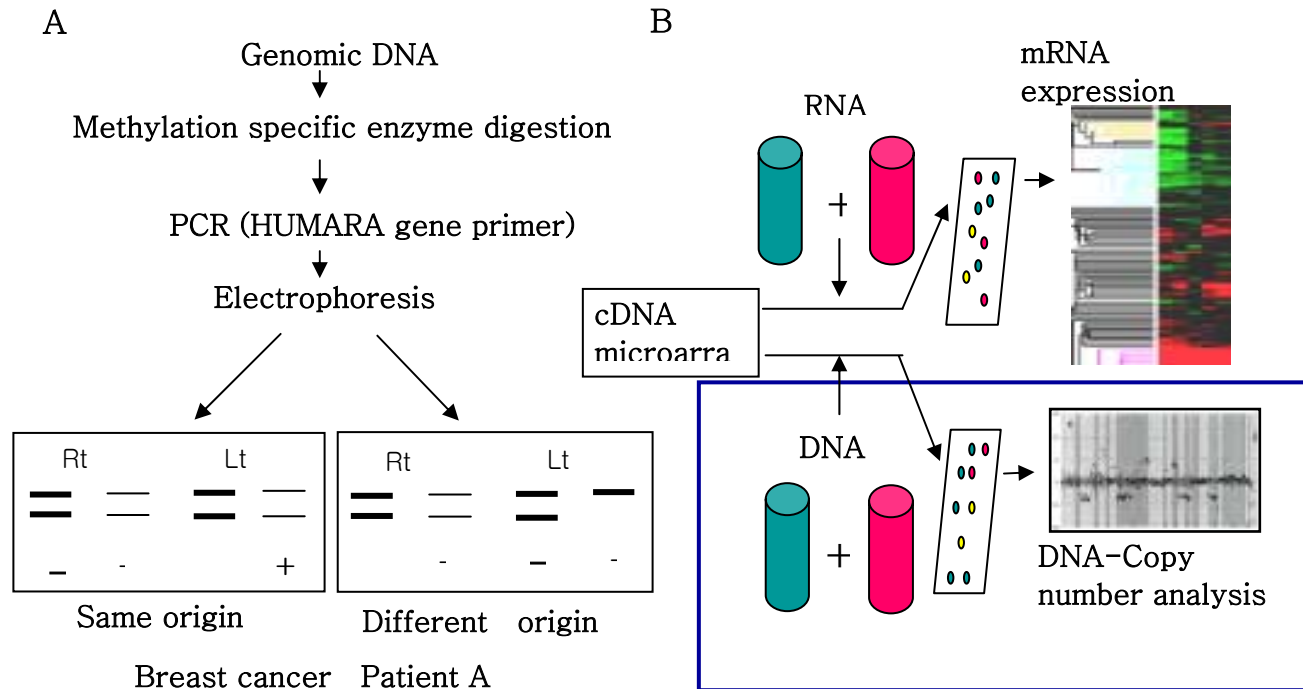
Fig.1 . Scheme of the study. A. X-chromosome inactivation pattern analysis. B. cDNA microarray based CGH pattern analysis.

Table 1. The scale of the study

	X- chronosome inactivation	cDNA array-based CGH
Control	2 Cancer Cell lines	2 Lymphocyte DNA from patients
Metachronous	5 pairs	3 pairs
Synchronous	10 pairs	3 pairs

2. X-chromosome inactivation analysis.

The status of activation of the X-chromosome in synchronous tumor and metachronous tumor cases was determined by using methylation - sensitive restriction enzymes, HhaI. A 280bp PCR amplification unit including the flanking HhaⅠsites and trinucleotide repeat element (nucleotides 229-508, HUMARA; Genebank) was desiginated for the human androgen receptor locus[4]. When the template DNA was digested with methylation sensitive restriction enzyme, the PCR amplification only occurred where the restriction sites had been methylated; otherwise, if any of the restriction sites were unmethylated, then amplification could not be successful due to the digestion with the flanking oligonucleotides binding region.

A. Cell culture

As a control for enzyme digestion and PCR, we used the YCC-2, YCC-6 gastric cancer cell lines which were established from the ascites of gastric cancer patients (Yonsei Cancer Center, Seoul, Korea). The cells were cultured and maintained in MEM with 10% fetal bovine serum (GIBCO, Grand Island, NY, USA), in 100 units/ml of penicillin and 0.1mg/ml of streptomycin (GIBCO, Grand Island, NY, USA) at 37℃, in a 5% CO_2 incubator.

B. Genomic DNA extraction

Dissected tissues from paraffin blocks, fresh frozen tissues and the cultured cells were incubated with 400ul of DNA lysis buffer [10mM Tris PH7.6, 10Mm EDTA, 50Mm NaCl, 0.2%SDS, 200ug/ml Proteinase K] at 42℃ for 12-24hours. The incubated products were boiled for 10 mins at 100℃ to inactivate enzymatic activity, and then treated with the same amount of phenol / chloroform / isoamylalcohol (GIBCO-BRL, Gaithersburg, MD, USA) to isolate the nucleic acid from the proteins. DNA was precipitated with 100% ethyl alcohol containing 1/3 volume of 10M ammonium acetate and 2ul of glycogen. After being rinsed with 70% ethyl alcohol, the DNA was dried at room temperature and then dissolved in ultra-pure water. DNA concentrations were determined using UV spectrophotometer at 260nm and DNA was stored at -20℃ until the experiment.

C. Enzyme digestion

For each DNA sample, two reactions were conducted simultaneously. One microgram of genomic DNA was digested with 20units HhaⅠ (Promega, Madison, WI, USA), and another 1ug of DNA was incubated in enzyme buffer without HhaⅠ enzyme. All reactions were conducted in a total volume of 20ul, and then incubated for 8-12h at 37℃. After the digestion, the reactions were terminated by boiling at 95℃ for 10 mins.

D. HUMARA-PCR assay[13]

Three micro liters of previous enzyme digested DNA was added to 30ul of the PCR mixture containing two oligonucleotide primers (Genotech, Daejun, Korea) at a concentration of 20 pmole, 250uM dNTPs (GIBCO-BRL, Gaithersburg, MD, USA), 0.5U *Taq* polymerase (GIBCO-BRL, Gaithersburg, MD, USA), 2.5mM MgCl$_2$, α-^{32}P dCTP and 3ul DMSO. The primers sequences were obtained from the previous report: primer 1, 5'--GCTGTGAAGGTTGCTGTTC-CTCAT--3' and primer 2, 5'--TCCAGAATCTGTTCCAGAGCGTGC--3' (Tilley et al. 1989). Samples were amplified for 28 cycles (45s at 95℃, 30s at 60℃ and 30s at 72℃) after the initial denaturation at 95℃ for 3mins in a thermocycler (MWG AGbiotech, Germany). Five micro liters of the PCR product was mixed with 5ul of 2X gel-loading buffer, and then the mixture was loaded into 6% 39:1 acrylamide / bis-acrylamide gel. Electrophoresis was performed at 80W for 3 hours. The gel was then dried and exposed to X-ray film (Kodak) at -70℃ for 12 hours using an intensifying screen. The gel was also stained with ethidium bromide and visualized under UV or stained with 0.1% silver nitrate.

3. cDNA microarray based genomic DNA hybridization.

A. cDNA microarray and control clones

Human cDNA microarrays (Genomic Tree Co, Daejun, Korea) containing sequence verified 974 genes were used. As spike

controls, 9 human cDNA clones were amplified using universal primer ; AI086446, AA903183, AI459073, AA490996, AA465697, AA457034, AA459263, AA28115 (Genebank ID).

B. Labeling and hybridization

The same genomic DNA used for the X chromosome inactivation assay were used as the test and reference samples. For each fluorescent labeling, we used 4ug of digested genomic DNA with DpnⅡ (New England Biolabs, Beverly, MA, USA), which was then purified QIAquick PCR purification kit (QIAgen, Dusseldorf, Germany). In case of the DNA from paraffin embedded tissues, fragmentation was not needed because it had already degraded (Fig.2). Fragmented DNA was random-primer labeled using a Bioprime Labeling kit (GIBCO-BRL, Gaithersburg, MD, USA). We modified the method[8] to allow a 50ul reaction, 10x low dCTP-dNTP mix (containing of 1.2mM each dATP, dGTP, dTTP and 0.6mM of dCTP; GIBCO-BRL, Gaithersburg, MD, USA) and Cy5-dCTP or Cy3-dCTP (0.6Mm ; DuPont NEN Life Sciences, Boston, MA, USA). The reactions mixtures were incubated at 37℃ for 2 hours in dark space. The reaction was stopped by adding 5ul of 0.5 M EDTA, pH8.0.

Pre-hybridization was performed with the blocking solution consisted of 3.5X SSC, 0.1% SDS, 10mg/ml BSA and dH$_2$O. The solution was filtered and incubated at 42℃ or 50℃ for 30 min-1 hour. The spotted slides were dipped in water and in isopropanol serially and then completely dried at 1000 rpm for 5mins.

Cy5- and Cy3- labeled probes were mixed with 30ug human Cot-1 DNA (GIBCO-BRL, Gaithersburg, MD, USA), 20ug poly (dA)-poly (dT) (Sigma, Saint Louis, Missouri, USA), and 100ug yeast tRNA (GIBCO-BRL, Gaithersburg, MD, USA). A Microcon-30 filter (Amicon, Bedford, MA, USA) was used to purify and concentrate the hybridization mixture, which was then adjusted to contain 3.4X SSC and 0.3% SDS in a final volume of 40ul. Following denaturation at 100℃ for 1.5 mins and a 30 mins of pre-annealing at 37℃, the probe was hybridized to the array under a glass coverslip at 65℃ for 24-30 hours. The probe was then washed in washing solutions with 0.5X SSC-0.01% SDS, 0.06X SSC-0.01% SDS, and 0.05X SSC three times at room temperature and dried by centrifugation at 1000rpm for 5 mins.

C. Imaging and data analysis

Hybridized arrays were scanned using a GenePix 4000B (Axon Instruments, USA) and fluorescence signals were calculated after subtracting the background by GenePix Pro 4.0(Axon Inc.USA). Poor feature signals (F532 nm-1.5 X B532 nm < 0 , F635nm-1.5 X B635nm < 0) were filtered out as flagging. An 'MA-plot'[12] was used to represent the (R, G) data, where $M = \log_2 R/G$ and $A = \log_2 (R \times G)^{1/2}$; R means F635 signal from Cy-5 and G means F532 signal from Cy-3 labeling. With MA-plots, we identified spot artifacts and detected intensity – dependent patterns in M for the purpose of normalization (Fig.3). To correct the differences originated from inter-sample DNA-labeling efficiency, a 'within–pin tip group

normalization' was performed[14]. A raw data was simply normalized relative to a (pin tip+A),

    i.e. $\log_2 R/G \rightarrow \log_2 R/G - c_i(A) = \log_2 R/[k_i(A)G]$

where $c_i(A)$ is the Lowess fit[14] to the MA-plot for the *i*th pin group only,*i*=1,2,….*I*, and *I* denotes the number of pin groups.
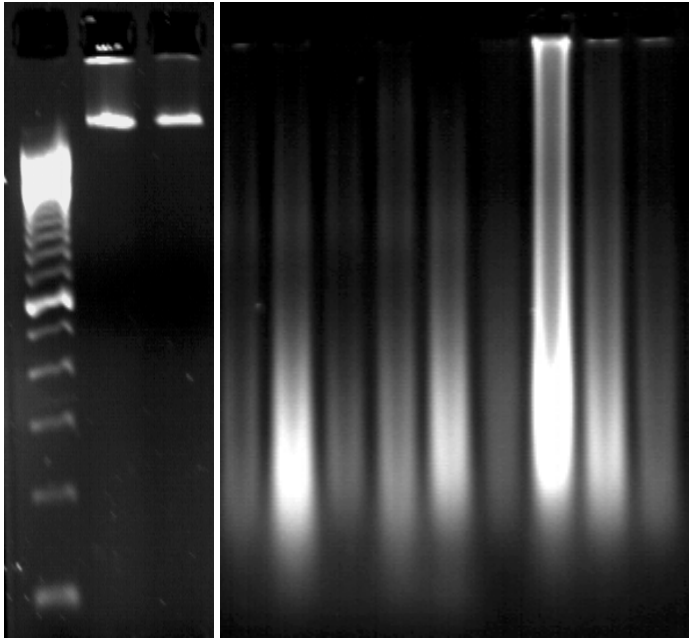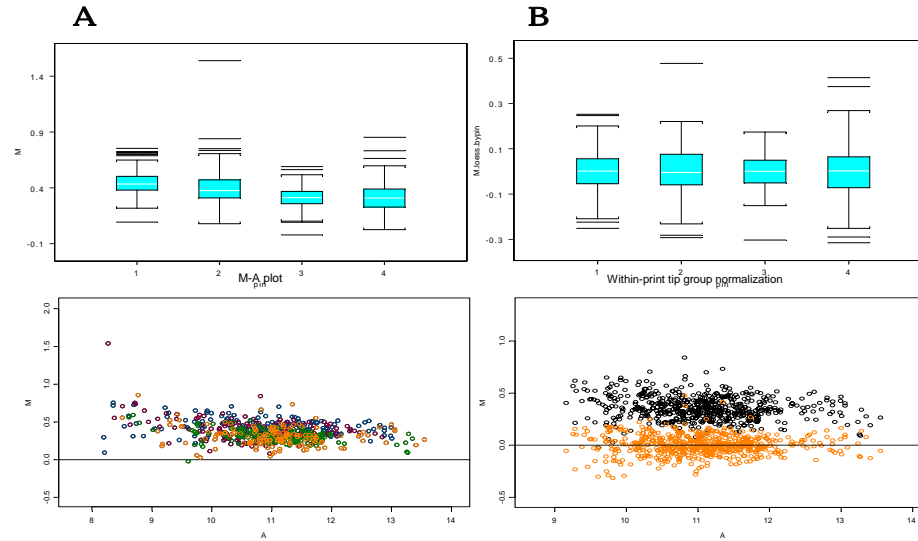
Fig. 2. Genomic DNAs. The left is genomic DNA from fresh frozen tissue and the right is genomic DNA from paraffin embedded tissue.

Fig.3.   MA- plots after within- pin tip normalization.
A. Before the normalization. B.After the normalization.

Ⅲ. RESULTS
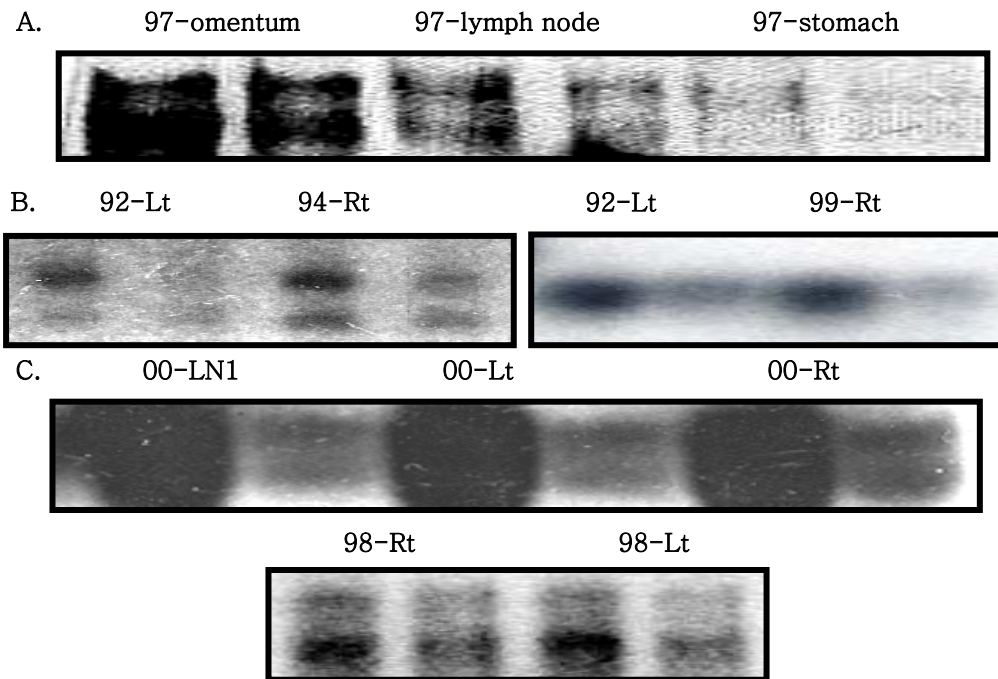
1. X-chromosome inactivation pattern analysis (XCIP)

XCIP was performed by using HUMARA-PCR assays, to determine the genetic origins in BBCs. We exploited XCIP analysis with 15 pairs of bilateral breast cancer specimen. We had expected different amplification pattern of HUMARA gene with one or two bands in BBC from different cancer origin (Fig.4). On the other side, in case of the pair of breast tumors originated from same clone, the band pattern would have been same[5, 6]. As a positive control for enzyme digestion and PCR, gastric cancer cell lines were tested for XCIP analysis. In case of the cell line from man, there were one allele, and the allele was disappeared after the enzyme digestion. Another cell line from woman, the band pattern was same as in BBC. We could evaluate the XCIP of gastric cancer tumor with ometum and lymph node metastasis from one patient. All of the three cases showed the same band pattern (Fig.5A). According to our XCIP results, there was a consistent pattern with paired samples regardless of their BBC type. Among five pairs of metachronous and ten pairs of synchronous tumors, each case showed the same band patterns in two comparative tissues (Fig. 5). This fact suggests that the XCIP analysis is not enough to discern the genetic origin of tumors in BBC with our small cases. Therefore, we then decided to perform a further analysis using array-based comparative genome hybridization (CGH).

Bilateral breast cancer　patient A

Same origin	Different origin
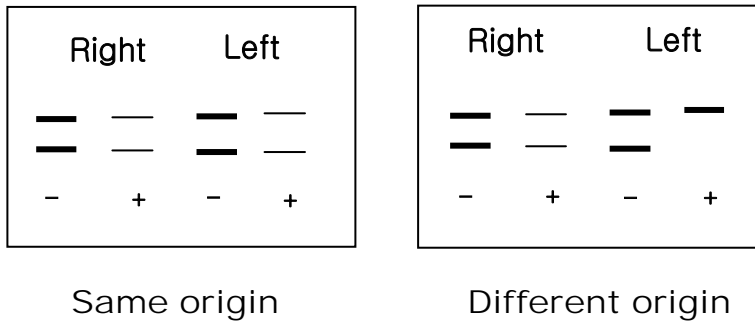
Fig4. Expected amplification patterns of HUMARA
gene in the pair of bilateral breast cancer.

Fig.5. X- chromosome inactivation pattern analysis in bilateral breast cancer A. stomach cancer with omentum and lymph node metastasis; B. metachronous bilateral breast cancer ; C. synchronous bilateral breast cancer. (First number indicates the year when the tumor was procured. LN- lymph node Rt- right breast cancer Lt- left breast cancer.
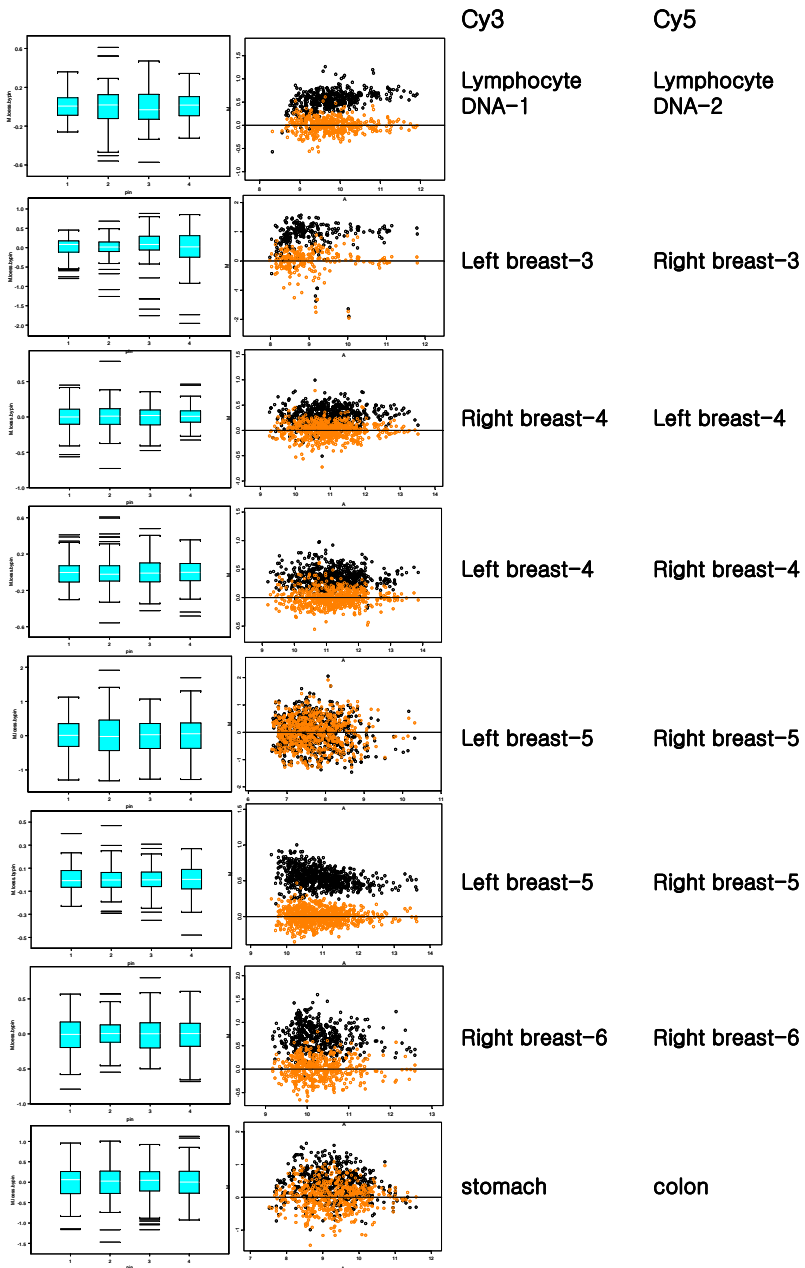
2. cDNA microarray-based comparative genomic DNA hybridization pattern analysis.
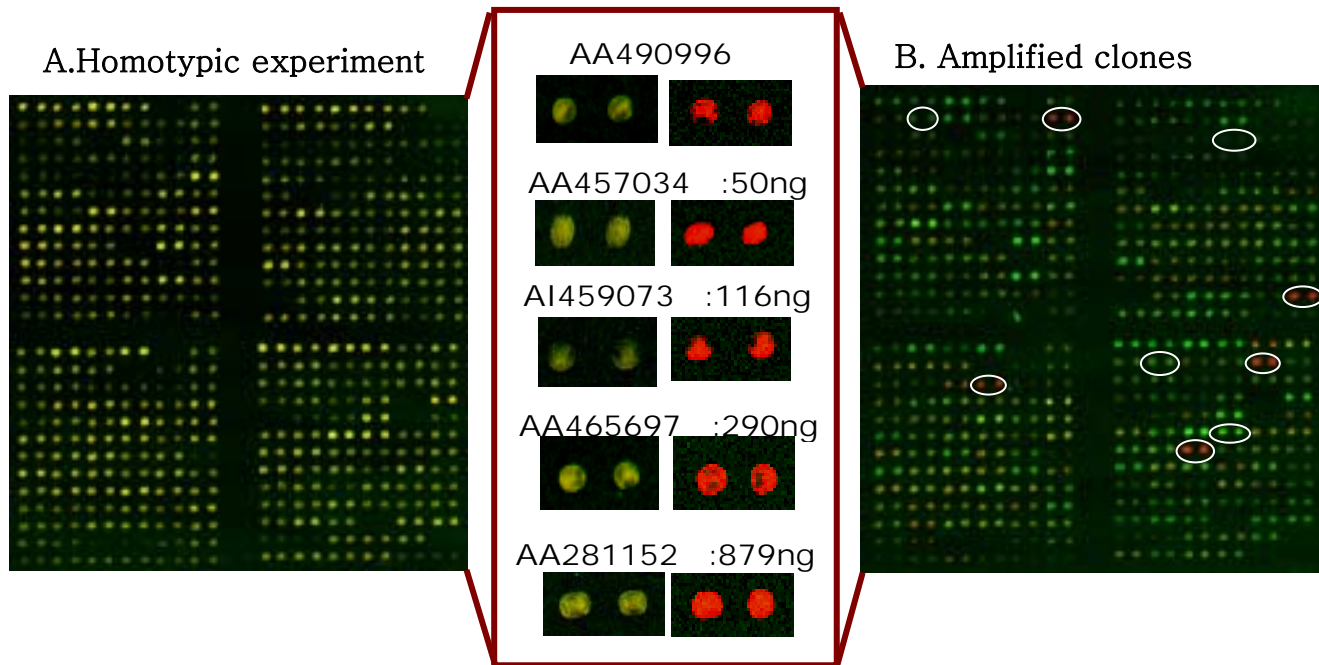
We compared genome-wide differences between individual tumors in BBC utilizing hybridization of the total genomic DNA onto cDNA microarrays. Data analysis was proceed after filtering and performing within-pin tip group normalization (Fig.3 and Fig.6).

At first, we confirmed the systematic reliability of CGH technique that we employed by doing the homotypic hybridization test using the same DNA labeled with cy3 or cy5 (Fig.7). When two different dyes were labeled with same DNA source and hybridized together, the average log ratio after homotypic hybridization was 0.0005 indicating no bias in our condition of the hybridization.

To evaluate the specificity of the hybridized spot signal on array-based CGH chip and to evaluate the experimental bias from dye labeling efficiency, samples from synchronous case was tested in dye swapping method (Fig.7). The genomic DNA from left breast tissue of one patient was labeled with cy3 and from right breast tissue of same patient was with cy5 and then reverse labeling was performed. An average log ratio for all spots in dye swapping test was 0.0028.

Cy3	Cy5
Lymphocyte DNA-1	Lymphocyte DNA-2
Left breast-3	Right breast-3
Right breast-4	Left breast-4
Left breast-4	Right breast-4
Left breast-5	Right breast-5
Left breast-5	Right breast-5
Right breast-6	Right breast-6
stomach	colon

Fig.6. Box plots and MA- plots after the normalization

A.Homotypic experiment

AA490996

AA457034   :50ng

AI459073   :116ng

AA465697   :290ng

AA281152   :879ng

B. Amplified clones

Fig.7.   Pseudocolour image of cDNA microarray hybridization of spikes control to confirm the specificity. A: Homotypic experiment. DNA of left breast tumor was labeled with Cy3 and Cy5 B: DNA from the left breast cancer was labeled with Cy3 and known DNA fragment which was amplified was labeled with Cy5.

Next, to evaluate whether the array-based CGH system recapitulate the changes of gene copy number, various amount of synthetic PCR products representing probes on array were exogenously added to hybridization targets. In figure 8, sample used in homotypic reaction was labeled with cy3 and amplified PCR products were labeled with cy5. We observed that 5 out of 9 exogenously introduced DNA fragments expressed significant signals on each spot (Fig. 8).

We tested genetic patterns of lymphocytes from two different patients. We assumed that, even in different individuals, most of the genomic DNA copy number for each gene must be quite similar to each other in healthy physiological condition. We observed that only 0.5% of genes out of 600 probes on array showed different DNA copy number. This result indicated that the expressions of certain genes are regulated at transcription and/or post-transcriptional level (Fig. 9).

In one hybridization assay with different type of tumor originated from the same patient (stomach vs colon cancer), notable log ratio values were observed in 28% of the spots in genomic DNA level suggesting that two samples showed significantly different genetic profiles (Fig. 10).
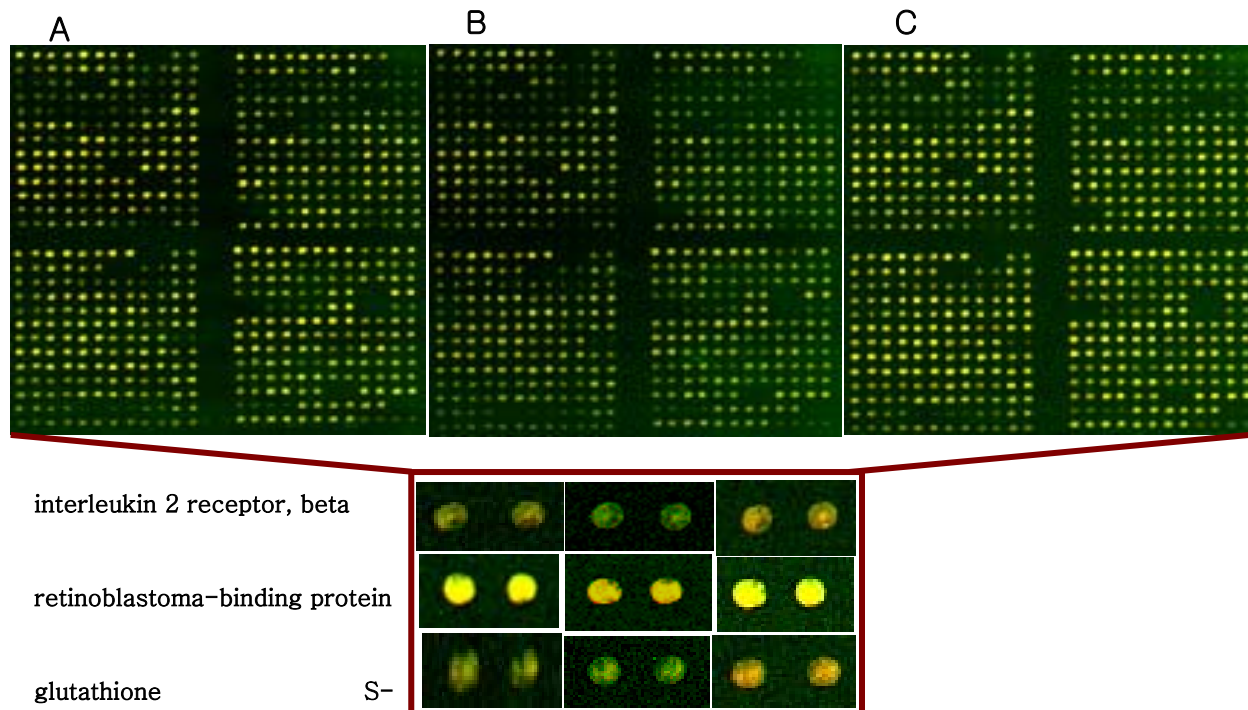
Fig.8. Synchronous bilateral breast cancer. A. Homotypic experiment. left breast tumor tissue was labeled with both Cy3 and Cy5. B. right breast tissue was labeled with Cy3 and left was labeled with Cy5. C .dye swapping
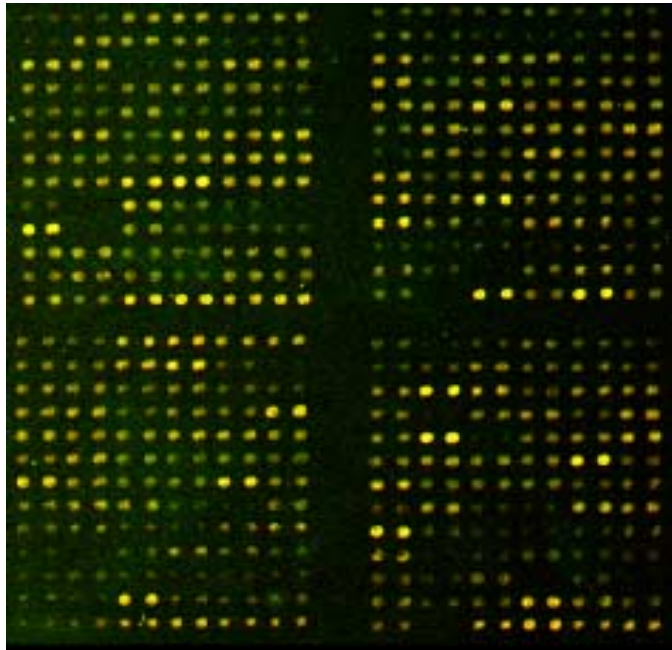
Fig.9. The comparison of genomic DNA from the lymphocyte of two different patients. Genes with different DNA copy number (over or under a log ratios were 0.5) are less than 0.5%.
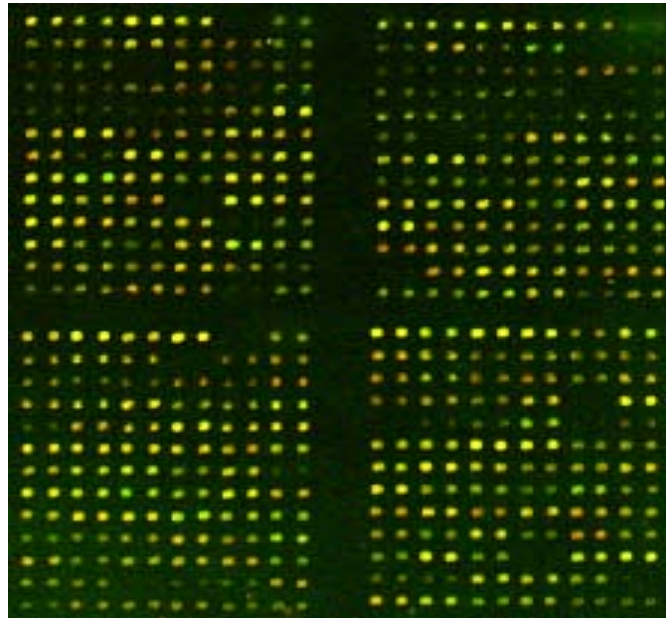
Fig.10. Comparison of the genetic patterns between stomach cancer and colon cancer in one patient. It can be used positive control. (cy3- stomach cancer vs. cy5- colon cancer tissue) It was validated by dye swapping tests.

We hybridized three pairs of synchronous tumors and fours pairs of metachronous tumors, respectively (Fig. 6, 11, 12, 13). Genomic DNA from one pair of three synchronous cases and two pairs out of four metachronous cases were used in both array-based CGH and XCIP analysis. Only one out of three synchronous cases, which did not show any different pattern in XCIP analysis, showed 4.2% meaningful log ratio pattern changes. But rests of them were close to 0 suggesting that two tumors had similar genetic profiles at the genomic DNA level (Fig. 6, 11, 12).

As expected, metachronous tumors were found to have more genes with copy number changes than synchronous tumor (Fig. 12, 13). This finding was similar to the previous result with 2 different tumor types in one patient (Fig. 14). Figure 14 represented significant pattern of genomic DNA changes between the metachronous and synchronous tumor in BBC. While the average log ratio values of the different genomic DNA change were 2% in synchronous pairs, it was 14.3% in metachronous pairs.

In two metachronous cases Thirty-nine genes changed had high log ratio values. The expression of constantly genes was simultaneously altered in all of the three metachronous BBCs (Table 2). These genes would be tumor progression related genes in breast cancer[18, 20]. Figure15 represents the chromosomal regions of altered 8 genes in metachronous tumors.

A

B
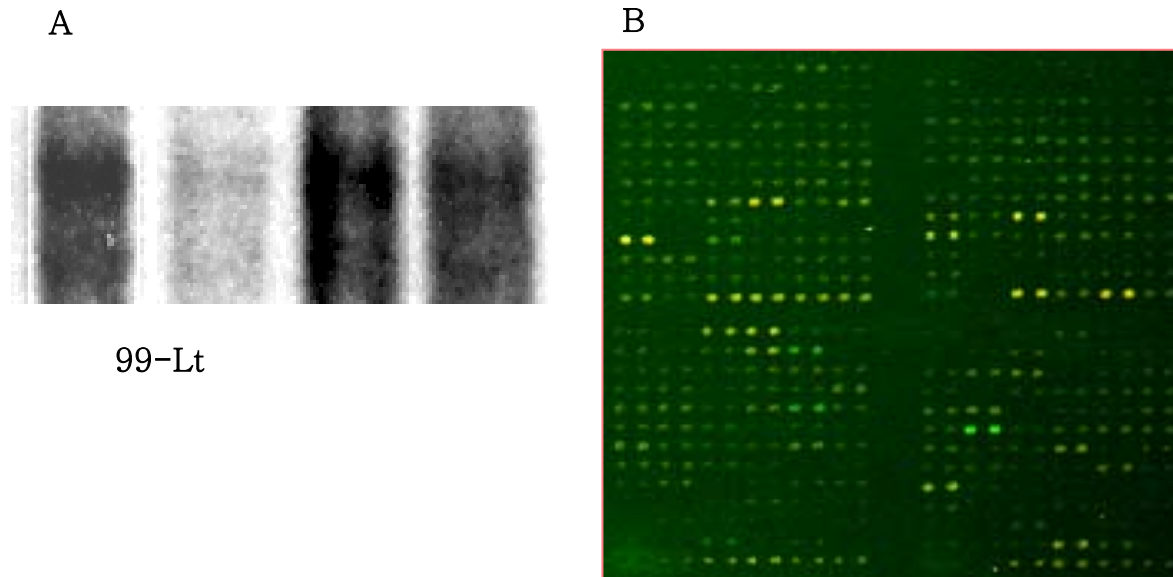
99-Lt

Fig.11. Comparison of the pattern analysis in synchronous BBC using the array based CGH and X chromosome inactivation assay. A. X- chromosome inactivation
pattern is similar in this BBC case. B. 99- Lt waslabeled with Cy3 and 99- Rt was labeled with Cy5. The result shows 4.2% meaningful genes with high log ratio values of the all spots.

A

A1-00-Lt   A2-00-Lt   A3-00-Lt   89-Rt

B.

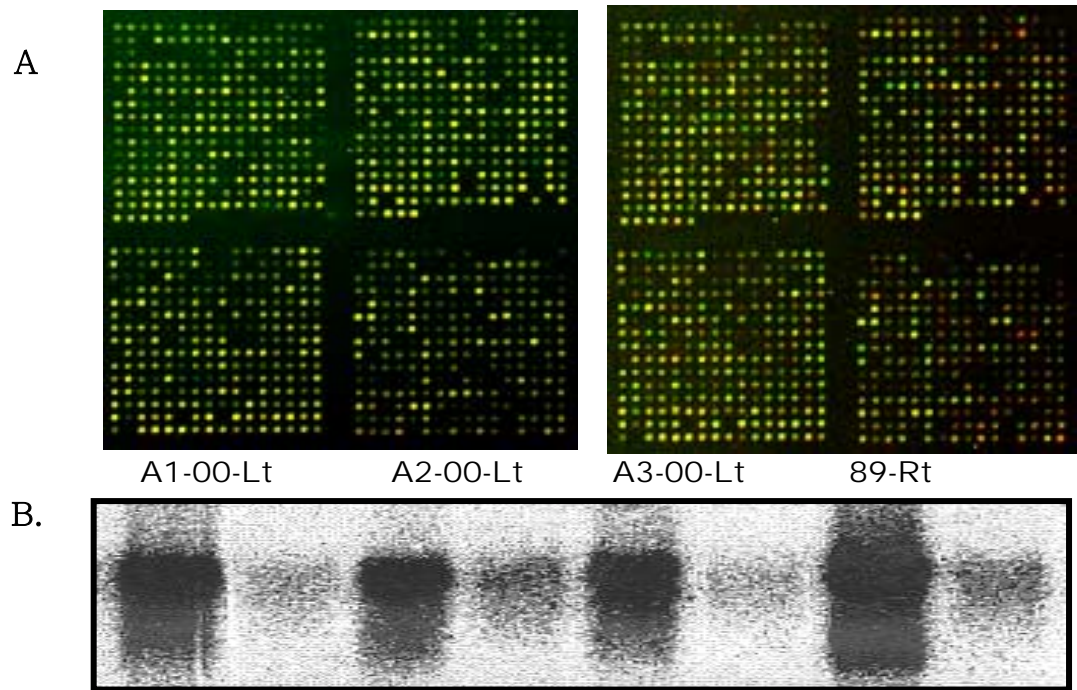Fig.12. Pattern analysis in synchronous and metachronous cancer with the array based CGH and X chromosome inactivation assay. A. Left ： A2-00-Lt was labeled with Cy3 and A3-00-Lt was labeled with Cy5 Right ： A2-00-Lt was labeled with Cy3 and 89-Rt was labeled with Cy5 B. X-chromosome inactivation pattern is similar. The HUMARA gene is homozygous in the samples. A1,A2,A3 were multi-focal tumors in left breast cancer.
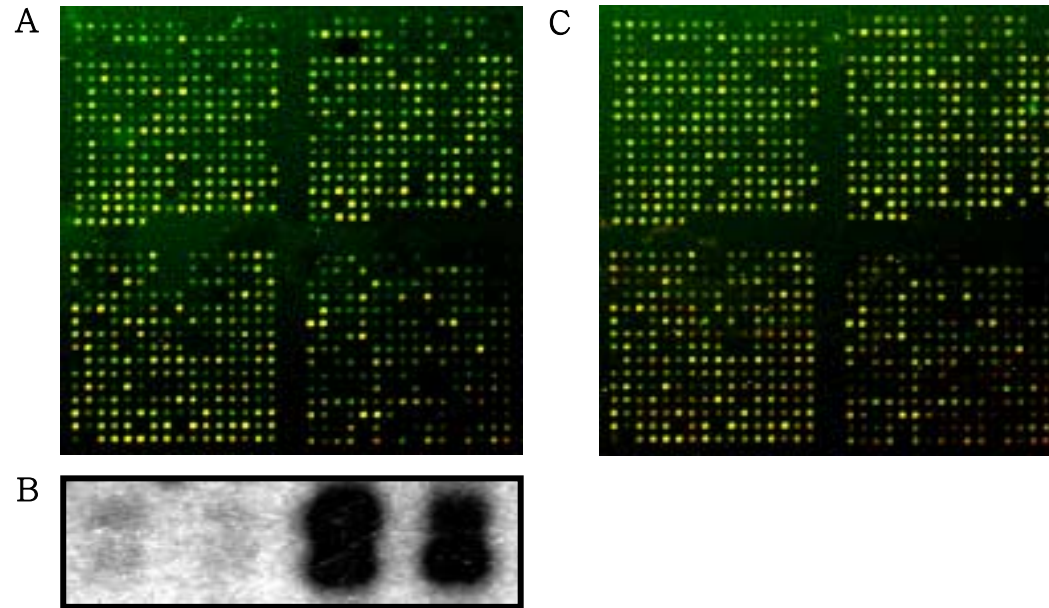
Fig.13.　A. 94-Left (Cy3)　98-Rt (Cy5)　　B. The pattern of X chromosome

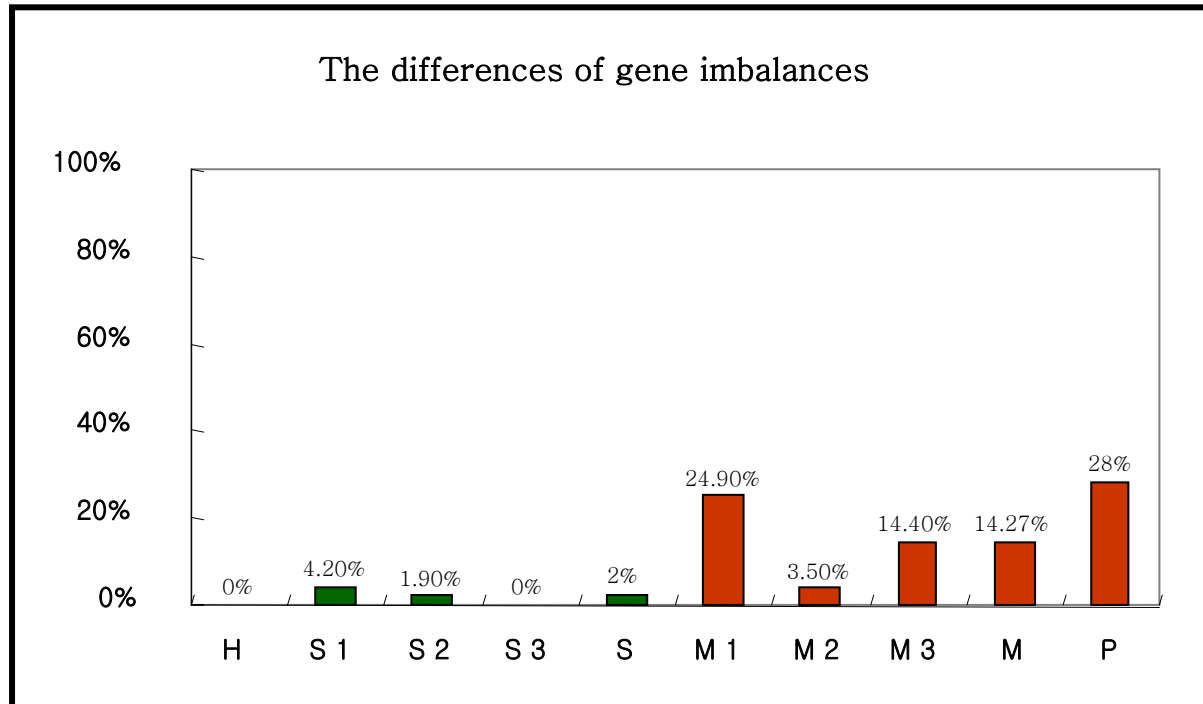inactivation in same individual with A. C. 01-Rt (Cy3) 96-Lt (Cy5)

Fig.14. Differences of the altered genes in BBC.
H:Homotypic hybridization, S:synchronous, M:metachronous, S1-S3: 3synchronous BBCs, S:average of S1,S2 and S3, M1-M3: 3metachronous BBCs, M:average of M1,M2 and M3, P: altered genes in 2 different tumor types.

Table 2. Genes of high log ratio values in three metachronous
cases simultaneously.

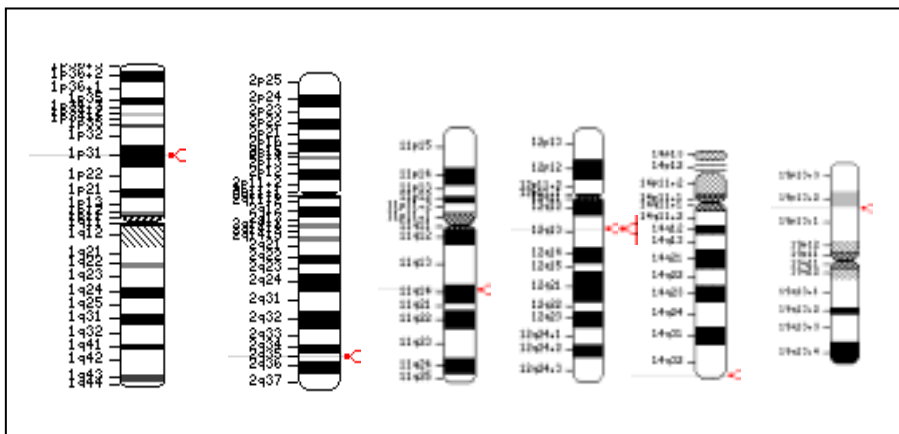| Name	ID
cathepsin C	AA644088
cystathionase (cystathionine)	R07167
erythropoietin receptor	H15574
pleckstrin homology, Sec7 and c	AA480859
quiescin Q6	AA464217
retinoic acid receptor, gamma	AA496438
wingless-type MMTV integration	AI884731
X-ray repair complement	AA775355

Fig.15. Chromosomal region of the simultaneously changed
genes in 3 metachronous cases in table2.

Ⅳ. DISCUSSION

Understanding of the clonality of BBC is very important for prognosis prediction and proper treatment. However, using current knowledge, the phenotypic features of BBC are hard to differentiate them in clinically or biologically. Empirically determined diagnostic criteria have been proposed for the discrimination of multiple primary and metastatic bilateral lesions. Although it is clinically accepted that BBC was originated from two clonally independent primary malignancies, molecular or genetic approaches to discern this clonal issues have not been accumulated enough to make any conclusion so far.

The inactivation of one of the two X chromosomes occurs early during development. This phenomenon resulted in individuals with cellular mosaics with either the maternal or paternal X chromosome inactivated[7, 11]. On the contrary to this, tumors from single cell origin have only one type of inactivated X chromosome. When the template DNA was digested with methylation sensitive restriction enzyme, the HUMARA PCR amplification only occurred where the restriction sites had been methylated; otherwise, if any of the restriction sites were unmethylated, then amplification could not successful due to the digestion with the flanking oligonucleotide binding region[8]. This concept could be applied to determine whether the tumor was originated from the same or different locus in BBC[5, 6].

In our case, the XCIP analysis was not informative in terms of discrimination of genetic origin for both synchronous and metachronous tumors. Thus, limited conclusions could be drawn from

36

our data, which implied the limitations of the X chromosome inactivation assay for the genetic characterization as we concerned. Since X chromosome inactivation occurs only one allele either from father side or mother side by selective DNA methylation, and selected X chromosome is inherited to daughter cells when they divide, it is possible that the same allele could be methylated in tumors developed from different clones. Consequently, the pattern analysis by XCIP may give us inaccurate information about the genetic origin of cancer[11]. Furthermore, the success of this test depends on the heterozygosity of the X-linked marker analyzed. It has been found that its significance may be blurred by the occurrence of an allelic imbalance at the X chromosome in breast tumor[16, 17]. The other point is the same pattern in a tumor may not be solely interpreted that the tumor was originated from one cell population. Indeed, single cell clone, or small number of cells, fortuitously inactivated at the same loci of X chromosome may outgrow during the process of neoplasia. It is also ambiguous to make a decision using this X chromosome pattern analysis due to increasing reports regarding the widespread methylation instability in cancer genome[11]. In addition, the amount and quality of DNA extracted from the archived tissues may influence on our results.

Gene amplification is one of the major mechanisms of oncogene activation in tumorigenesis. On the other hand, inactivation of tumor suppressor genes by loss of heterozygocity (LOH), CpG island methylation is also important genetic and epigenetic mechanism of tumorigenesis. With the development of the technique of CGH onto

microarrayed cDNA clones[12, 13], it provides us more high-through powerful analyses for identifying and mapping the altered genes, which are assumed as highly disease-related genes. This approach is particularly attractive because of the availability of thousands of accurately mapped cDNA [18, 19]. Our results showed that the array-based CGH could potentially be used as a comparative analysis tool for genetic characterization in tumors when their originality is not clear as in bilateral breast cancers.

To confirm this modified new techniques, we performed the five basic experiments (Fig. 7, 8, 9, 10). When homotypic experiment was performed, theoretically we must have the same signal intensity after hybridization for each annotated DNA probe. Therefore, an average log ratio of signal intensity (log_2Cy5/Cy3) should be zero, which means the tested DNA copy number was same. If the range is out of acceptable range (0±0.5), then we may suspect that the array hybridization results had a dye bias or experimental errors (Fig. 7).

In figure 8, we showed the specificity of the hybridization using 9 amplified spikes. As we expected, the spiked DNA yielded very strong intensity, whereas genomic DNA level in counterpart (cy3) displayed same pattern with previous experiments. Thus, this result suggested that our array-based CGH system can specifically recapitulate the genomic changes in target preparation. Although we expected that all of the 9 spikes express significant signals, five of nine amplified clones expressed the specific signal. Since we used the mixture with various diluted amounts of the test spike DNAs to total amounts of 2ug, one clone was too small to cover the while diluted spots. We also figured

out that the rest 3 clones had lots of restriction sites for the enzyme DpnII and were fragmented *in silico* experiments simulating enzyme digestion (http://tools.neb.com/NEBcutter). These fragments might produce nonspecific signals.

Taken together with these results, although XCIP analysis did not provide enough information for genetic origin analysis in bilateral breast cancer, genetic profiling using array-based CGH supports the informative evidence to find difference of the genetic origin in tumors. Our results indicated that synchronous tumors presenting with highly concordant genetic profiles may correspond to contralateral metastasis. On the other hand, metachronous tumors exhibiting different patterns can be the secondary primary tumor. A similar pattern with additional abnormalities may fit the model of metastatic origin[24]. However, for clinical application, these should be investigated further by using more large scale approaches.

Ⅴ. CONCLUSION

Studies based on X chromosome inactivation produced partial information about the evidence of the genetic origin of selected bilateral breast cancer cases. We have demonstrated that the cDNA array-based CGH approach presented here could be a useful tool for detecting the genetic origin in tumors by comparing chromosomal abnormality patterns, and thus discern the genetic origin in case of bilateral breast cancer.

Ⅵ. REFERENCE

1. Shibata A, Tsai YC, Press MF, Henderson BE, Jones PA, Ross RK. Clonal analysis of bilateral breast cancer. Clin Cancer Res 1996; 2: 743-8

2. Jack H. Lichy, Fabienne Dalbe`gue, Maryam Zavar, Constance Washington, Mark M. Tsai, Zong-Mei Sheng, et al. Genetic heterogeneity in ductal carcinoma of the Breast. Lab Invest 2000; 80: 291-301

3. Susan R. Harris. Second-guessing a second primary. CMAJ 1999; 161: 1151-3

4. Coradini D, Oriana S, Mariani L, Miceli R, Bresciani G, Marubini E, et al. Is steroid receptor profile in contralateral breast cancer a marker of independence of the corresponding primary tumor? Eur J of Cancer 1998; 34: 825-30

5. Noguchi S, Motomura K, Inaji H, Imaoka S, Koyama H. Clonal analysis of solitary intraductal papilloma of the breast by means of polymerase chain reaction. Am J Pathol 1994; 144: 1320-5

6. Zhuang Z, Lininger RA, Man YG, Albuquerque A, Merino MJ, Tavassoli FA. Identical clonality of both components of mammary carcinosarcoma with differential loss of heterozygosity. Mod Pathol 1997; 10: 354-62

7. Vogelstein B, Fearon ER, Hamilton SR, Preisinger AC, Willard HF, Michelson AM, et al. Clonal analysis using recombinant DNA probes from the X-chromosome. Cancer Res 1987; 47: 4806-13

8. Allen RC, Zoghbi HY, Moseley AB, Rosenblatt HM, Belmont JW. Methylation of HpaII and HhaI sites near the polymorphic CAG

repeat in the human androgen-receptor gene correlates with X-chromosome inactivation. AJHG 1992; 51: 1229-39

9. Diaz-Cano SJ, Blanes A, Wolfe HJ. PCR techniques for clonality assays. Diagn Mol Pathol 2001; 10: 24-33

10. Kim NG, Roh JK, Kim JH, Chung WY, Park CS, Kim H. Clonality analysis using methylation-specific polymerase chain reaction: a novel method for investigating tumor clonality. Lab Invest 1999; 79: 1727-39

11. Sakurazawa N, Tanaka N, Onda M, Esumi H. Instability of X chromosome methylation in aberrant crypt foci of the human colon. Cancer Res 2000; 60: 3165-9

12. Pollack JR, Perou CM, Alizadeh AA, Eisen MB, Pergamenschikov A, Williams CF, et al. Genome-wide analysis of DNA copy-number changes using cDNA microarrays. Nat Genet 1999; 23: 41-6

13. Busque L, Zhu J, DeHart D, Griffith B, Willman C, Carroll R, Black PM, Gilliland DG. An expression based clonality assay at the human androgen receptor locus(HUMARA) on chromosome X. Nucleic Acids Res 1994; 22: 697-708.

14. Dudoit S, Yang YH, Callow MJ and Speed TP. Statistical methods for identifying genes with differential expression in replicated cDNA microarray experiments. Stat Sin in press 2002

15. Xu J, Meyers D, Freije D, Isaacs S, Wiley K, Nusskern D, et al. Evidence for a prostate cancer susceptibility locus on the X chromosome. Nat Genet 1998; 20:175-9

16. Dillon EK, de Boer WB, Papadimitriou JM, Turbett GR. Microsatellite instability and loss of heterozygosity in mammary carcinoma and its probable precursors. Br J Cancer 1997; 76: 156-62

17. Jain AN, Chin K, BorresenDale AL, Erikstein BK, Eynstein Lonning P, Kaaresen R, et al. Quantitative analysis of chromosomal CGH in human breast tumors associates copy number abnormalities with p53 status and patient survival. Proc Natl Acad Sci 2001; 98: 7952-7

18. Monni O, Barlund M, Mousses S, Kononen J, Sauter G, Heiskanen M, et al. Comprehensive copy number and gene expression profiling of the 17q23 amplicon in human breast cancer. Proc Natl Acad Sci 2001; 98: 5711-6

19. Murray AE, Lies D, Li G, Nealson K, Zhou J, Tiedje JM. DNA /DNA hybridization to microarrays reveals gene-specific differences between closely related microbial genomes. Proc Natl Acad Sci 2001; 98: 9853-8

20. Kollias J, Man S, Marafie M, Carpenter K, Pinder S, Ellis IO, et al. Loss of heterozygosity in bilateral breast cancer. Breast Cancer Res Treat 2000; 64: 241-51.

21. Wilhelm M, Veltman JA, Olshen AB, Jain AN, Moore DH, Pres, et al. Array-based comparative genomic hybridization for the differential diagnosis of renal cell cancer. Cancer Res 2002; 62: 957-60

22. Heiskanen MA, Bittner ML, Chen Y, Khan J, Adler KE, Trent JM, Meltzer PS. Detection of gene amplification by genomic

hybridization to cDNA microarrays. Cancer Res 2000; 60: 799-802

23. Adam N. R, Andy R, Deborah H, Ian J.J. Molecular evidence of a common clonal origin and subsequent divergent clonal evolution in vulval intraepithelial neoplasia, vulval squamous cell carcinoma and lymph node metastases. Int J Cancer 2002; 99: 549-54.

24. Siu Tim Cheung, Xin Yuan Guan, San Yu Wong, Lai Shan Tai, Irene O.L.Ng, Samuel So, et al. Identify Metastasis-associated genes in hepatocellular carcinoma through clonality delineation for multinodular tumor. Cancer Res 2002; 62: 4711-21

국문요약

양측성 유방암에서 genetic origin 의 동정

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서민영

양측성 유방암은 발생한 시간적 차이를 기준으로 synchronous tumor 와 metachronous tumor 로 나누어 질 수 있다. 각각의 경우에 양측성 유방암에서 암세포의 clonality 를 구분하는 것은 예후를 예측하고 치료에 이용하기 위해 매우 중요하다. 그러나 synchronous 종양과 metachronous 종양에서 각 종양이 한 원발암에서 유래한 전이 암인지 각기 독립적인 다른 clone 에서 유래된 종양인지를 정확하게 증명하기는 어렵다.

본 연구에서는 metachronous, synchronous 양측성 유방암에서 genetic origin 구분하기 위하여 분자 생물학적인 접근을 시도하였다.

먼저, 기존에 이용되었던 x chromosome inactivation assay 를 이용하여 분석해 보았으나, 이 방법은 결과를 분석하는데 나타나는 오류와 여성에게만 이용할 수 있는 등의 몇 가지 한계가 있었다.

이를 보완하기 위해 array-based genomic DNA hybridization 을 수행하고 결과를 분석하였다. 본 실험에 앞서 homotypic experiment, dye swapping tests, spikes hybridization 등의 실험을 통해 array- based CGH 에서 유전자 특이적인 hybridization 의 정확성, 재현성이 있음을 증명하였다.

Array-based CGH 결과는 metachronous tumor 3 예에서 synchronous tumor 3 예에서 보다 많은 유전자 변화가 있음을 보여주었다.

궁극적으로, synchronous 또는 metachronous 양측성 유방암에서 서로 다른 기원을 가진 암 조직은 genomic DNA 발현 패턴이 다르며, 이러한 유전자 패턴 분석 방법을 이용하여 전이에 의한 재발인지, 이차적 원발 종양인지를 구분할 수 있는 가능성을 제시 하였다.

핵심 되는 말: Genetic Origin, 양측성 유방암, X-Chromosome Inactivation Pattern Analysis, Array-based Comparative Genomic DNA Hybridization Analysis