Pharmacogenomic markers predicting toxicity and response of gemcitabine in breast cancer patients

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Pharmacogenomic markers predicting toxicity and response of gemcitabine in breast cancer patients

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The Master's Thesis submitted to the

Department of Medical Science,

the Graduate School of Yonsei University

in partial fulfillment of the requirements for the degree of

Master of Medical Science

Yeon Ho Choi

June 2005

This certifies that the Master's Thesis of

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June 2005

ACKNOWLEDGEMENTS

포장된 도로보다 투박한 시골길로 도착하고도 남았을 거리를 지금에서야 이 자리에까지 오게 되었습니다. 남보다 늦은 감이 있지만 후회라는 단어만큼은 떠올리고 싶지 않게 보낸 시간 이었습니다. 이런 저에게 다시 시작할 수 있는 기회를 주시고, 지도해 주신 푸근한 정현철 선생님 그리고 라선영 선생님은 아마 잊지 못할 것 입니다. 저의 관심 분야에 조언을 주시고 sample 까지 보여주셨던 양우익 선생님, 항상 저랑 같이 있어 손해 본다고 하시지만 매번 도움을 주신 정희철 선생님, '배워 익히기에 힘써 네 이치의 길잡이가 되라'고 말씀하셨던 서울대 박상대 선생님 실수투성이인 저를 받아주시고 실험실 생활의 모든 초석을 다지게 해주셨던 제 평생의 선생님이십니다. 쓴 소리 마다 않으셨던 항상 웃음짓던 서울대 김재범 선생님 새벽 녘까지 백세주 사주시고 다시 실험실 가셔서 논문 쓰시던 그 모습은 잊을 수 없는 추억이었습니다. 처음 cloning을 통해 분자생물학을 시작할 수 있는 동기를 주셨던 최인순 선생님 제가 아는 모든 선생님들께 감사 드립니다.

실없는 저의 농담과 짜증 이 모든 것을 다 받아준 친구들에게도 고맙다는 말을 전합니다. Utah에서 열심히 박사 과정 공부를 하고 있는 내 의지와 상관없이 항상 보고픈 언주, 새로운 길에 도전한 멋진 친구 윤자, 굳이 말을 하지 않아도 이 세상 가장 편한 대화를 한 것 같은 25년 지기 친구인 수진이, 고딩 칭구 은영이, 경아, 미선이, 멋진 백대위 백호 호영이, 윤희, 승희, 희영, 200만 화소를 사랑하는 선옥, 내 친구 같다고 여겨지지 않는 이쁜 대학 동기들 안지. 쏘정, 수연, 연정, 혜경, Texas로 간 선진, 실험실 생활의 첫 번째 황금 멤버

선아 언니, 삼미, 미경, 승하, 은영, 씩씩이 혜정, 잊을 수 없는 서울대 세포생물학 실험실 분들, 내 실험실인양 들락거려도 항상 반겨주는 김주항 선생님 실험실 부원인 도통 시간 개념 없는 유박사 지영이, 재성 오빠를 대표로 모든 부원에게도 감사하다는 말을 전합니다. 현재 진행형인 연세대 암전이연구센터 실험실 부원들과 박규현, 김태수 선생님, 스쳐갔던 세나, 상택, 주현, 효영 등 감사하단 말을 전합니다. 그 중에 나를 가장 가까이서 지켜본 박군 아니 박박사 찬희 언니, 유부 우렁 오빠, 주혜 언니, 새롭게 합류한 정수 정말 고맙습니다. 준 것보다 받은 것이 너무 많아 참 행복한 사람인 것 같습니다.

무엇보다도 못난 딸 여기까지 오는데 아무 말 없이 그저 지켜만 보셨던 저의 부모님 최정우, 김석란씨, 아마 살아온 평생 동안 고맙다고 사랑한다고 말해본 적 없는 무뚝뚝한 딸인 저를 여기까지 그저 바라만 보시고 믿고 지켜봐 주신 부모님께 감사합니다. 사랑스런 우리 할머니 재희씨, 든든한 185 ㎝ 의 나의 낭군들 지영이, 지훈이도 모두 사랑합니다.

꿈이라는 것은 단지 꾸는 존재이기보다 이루어질 존재라고 생각합니다. 살면서 미쳤다는 말을 들어보지 못했다면 단 한번도 목숨 걸고 도전한 적이 없는 것이라고 합니다. 꿈을 이루기 위한 첫 도전인 제 논문이 나오기까지 너무 긴 시간이 흐른 것 같습니다. 흐르는 시간 속에서 익혀온 조그만 지식들을 토대로 제 자신을 발전시켜 좋은 길잡이가 되도록 하겠습니다.

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ABSTRACT

Pharmacogenomic markers predicting toxicity and response of gemcitabine in breast cancer patients

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We examined the pattern of single nucleotide polymorphisms (SNPs) of gemcitabine metabolism-related genes in breast cancer, and evaluated its possibility as a predictive marker for drug response and

toxicity. SNPs in deoxycytidine kinase (dCK), deoxycytidine monophosphate deaminase (DCTD), and ribonucleotide reductase M1 polypeptide (RRM1) were analyzed with genomic DNA of 10 breast cancer cell lines, 76 peripheral blood mononuclear cells (PBMC) from advanced breast cancer patients treated with gemcitabine, and 56 PBMC from healthy volunteers using CEQ^{TM} 8000 Genetic Analysis System (Beckman). The incidences of SNPs of breast cancer patients were 1.4 % of dCK (626 A>G), 10.8 % of DCTD (315 T>C), 40.5 % of the 1st RRM1 (2455 A>G), 44.6 % of the 2nd RRM1 (2464 G>A) and 23 % of both RRM1 sites, which were similar to normal control group. Especially, we found the 2 SNP of RRM1, 2455 A>G and 2464 G>A to be the novel sites. We observed that the variations in both RRM1 sites were associated with neutropenia ($p < 0.01$) and G-CSF requirements ($p < 0.005$). In addition, 3 out of haplotypes of RRM1 were significant based on Hardy-Weinberg equation. Meanwhile, no genotype did show an association with the tumor response.

IC_{50} of gemcitabine in 10 breast cancer cell lines were determined using MTT assay. Comparing the pattern of SNPs with IC_{50} of gemcitabine, we observed the 8 cell lines with RRM1 (2464 G>A) variation showed increased sensitivity to gemcitabine. Also, we observed the 8 cell lines with RRM1 variation showed decreased mRNA and protein expression level of RRM1.

In conclusion, RRM1 genotyping in PBMC might be a useful biomarker to predict the toxicity and the response of gemcitabine monotherapy in breast cancer patients.

Key Words : Gemcitabine, Single Nucleotide Polymorphism (SNP), DCTD (deoxycytidine monophosphate deaminase), dCK (deoxycytidine kinase), RRM1 (ribonucleotide reductase M1 polypeptide), Pharmaco-genomics

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I. INTRODUCTION

Pharmacogenomics aims to identify the inherited bases (age, race, organ function, gender, drug interaction) for inter-individual differences

in drug response and toxicity with using genome wide approaches.[1] Pharmacogenomics combines traditional pharmaceutical science with an understanding of common changes in the human genome.[2] On the contrary, pharmacogenetics aims to identify genetic polymorphisms that govern an individual' s response to specific drugs.[3] Clinical observation of inherited difference in drug effect was first documented in the 1950s, giving rise to the field of pharmacogenetics and later, pharmacogenomics.[4] One of the common variations in the human genome is single-nucleotide polymorphisms (SNPs). More than 1.4 million SNPs were identified in the initial sequencing of the human genome with over 60,000 of which in the coding region of genes. Some of these SNPs have shown to be associated with substantial changes in the metabolism or in the effect of anticancer agents, and some are now being applied to predict clinical outcomes.[5-9]

Gemcitabine (2' 2-difluorodeoxycytidine, dFdC) is a deoxycytidine analogue to Ara-C. It is a novel anticancer agent that has significant

activity in carcinomas of the ovary, lung, pancreas, and breast.[10] Gemcitabine has a complex metabolic pathway for cytotoxicity.[11,12] It is transported into the cell by nucleoside transporters, and phosphorylated by deoxycytidine kinase (dCK) to its active monophosphate form.[13,14] Therefore, dCK plays a key role in the activation of gemcitabine and its activity correlates with drug sensitivity.[15-20] Gemcitabine is inactivated by deoxycytidine monophosphate deaminase (DCTD) into its inactive form of difluorodeoxyuridine (dFdU). The deamination product of difluorodeoxycytidine monophosphate (dFdCMP) and difluorodeoxy uridine mono phosphate (dFdUMP) inhibit thymidylate synthetase (TS).[12] And the inhibition of TS activity is associated with an increase in DNA synthesis errors that lead to DNA damage. Ribonucleotide reductase is the rate-limiting step in DNA synthesis, because it is the only known enzyme that converts ribonucleotides to deoxyribonucleoside which are required for DNA polymerization and repair.[21] Ribonucleotide reductase is a holoenzyme which consists of dimerized ribonucleotide reductase

subunit 1 and 2 (RRM1, RRM2). RRM1 has been shown to function with p53 regulated RRM2 homologue p53R2, which is important in DNA repair secondary to genotoxic stress.[22]

In *in vitro* study, increased RRM1 expression and activity have shown to be a marker for gemcitabine resistance.[23-28] In this study, with the development of a rapid and practical method of genetic polymorphism analysis using automated CEQ ^{TM} 8000 Genetic Analysis System, we detected polymorphisms of dCK, DCTD and RRM1 in advanced breast cancer patients who received gemcitabine monotherapy. After correlation study between clinical response and toxicity with polymorphisms, we found the genotype of RRM1 to be a biomarker of toxicity in gemcitabine treatment.

II. MATERIALS AND METHODS

1. Patients and healthy volunteers

Total of 74 patients treated at Yonsei Cancer Center as advanced breast cancer were enrolled for the study. Patients eligible for this study had histologically confirmed breast cancer with documented progression after prior uses of anthracyclines and taxanes. All patients were required to be with at least single bidimensionally measurable lesion, to be 75 years of age or younger, to have an ECOG performance status of ≤ 2, and expected survival time of > 12 weeks, and to have adequate bone marrow, renal and hepatic functions (hemoglobin > 10.0 g/dl, leucocyte > 3,000 /mm^3, platelet > 100,000 /mm^3, total bilirubin and serum creatinine < 1.5 X of upper normal limit (UNL), and AST/ALT < 2 X UNL). Prior therapy must have been completed at least 4 weeks before study entry with full resolution of toxicities. Informed consent was required to all the patients according to the institutional regulations.

As a control, 54 healthy unrelated female blood donors were included in the study. Patients' characteristics are summarized in Table 1.

2. Treatment plan

Chemotherapy consisted of single dose of gemcitabine 850 mg/m^2 on D1, D8, and D15 every 28 days. Gemcitabine was mixed with 100ml normal saline and was infused intravenously for 60 minutes without premedication. Every week, patients had to have acceptable WBC (>3,000/mm^3) and platelet (>100,000/mm^3) counts to proceed next course of chemotherapy, and if not, the treatment was delayed on a weekly base until recovery. Dose reduction was not permitted, and if leucopenia had occurred (WBC < 3,000/mm^3), G-CSF was injected. Chemotherapy was given until disease progression, with unacceptable toxicity, or by treatment withdrawal. If the patient did not show definite evidence of disease progression, treatment was continued until 12 cycles.

3. Assessment of response and toxicity

Baseline evaluations included, physical examination, complete blood count (CBC) with the differential, serum chemistry, urine analysis, and electrocardiography. Antitumor activity was evaluated every 3 courses on all measurable diseases, and all the patients were scheduled for at least one course of treatment to be evaluable except for early progression. Tumor response was classified according to WHO criteria. If a patient was documented to have clinical response, a confirmatory was performed at least 4 weeks after the first documented tumor response. Time to progression (TTP) was calculated from the start of the treatment until progression, and overall survival (OS) was calculated from the starting day to death. Response duration was measured from the initial response until progressive disease. Toxicity was checked every course of treatment and evaluated by NCI-CTC version II.

4. Cell lines and cell culture

Human breast cancer cell lines (MCF/ADR, MDA-MB-231, MDA-MB-435, MCF-7, T47D, SK-BR-3,) were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland, USA). We also added another 4 cell lines, YCC-B1, YCC-B2, YCC-B3, and YCC-B5, which had been established from Korean breast cancer patients (Cancer Metastasis Research Center, Seoul, Korea). The cells were cultured and maintained in MEM supplemented with 10 % fetal bovine serum (GIBCO, Grand Island, NY, USA), 100 units/ml of penicillin, and 0.1 mg/ml of streptomycin (GIBCO, Grand Island, NY, USA) in a humidified 37 ℃ incubator with 5 % CO_2 in air.

5. Drug sensitivity test by 3-(4, 5-dimethylthazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) assay

The MTT assay was used to evaluate the sensitivity of cells to gemcitabine. $1x10^4$ cells were inoculated into each well of a 96-well

plate with various concentrations of gemcitabine. The plates were incubated for 72 hr and then 5 ㎍ of MTT was added to each well and the plates were incubated for an additional 3 hr. The resulting formazan was dissolved with 100 ㎕ 2-propanol containing 0.3 % HCl and the plates were shaken for 5 min with a plate shaker, and then read immediately at 570 nm using a model 550 Micro Plate Reader (Bio-Rad, Richmond, CA, USA). The assays were performed at least 3 times with triplicated samples.

6. Genomic DNA extraction

A. Cell line DNA preparation

The cultured cells were resuspended with 500 ㎕ of DNA lysis buffer [10 mM Tris-HCl (pH 7.6), 10 mM EDTA, 50 mM NaCl, 0.2 % SDS, 20 mg/ml Proteinase K] and incubated overnight at 42 ℃. After adding 500 ㎕ of phenol:chloroform:isopropanol alcohol (Gibco-BRL, USA), the samples were vortexed and then centrifuged at 4 ℃, 14,000 x g for 10

min. The upper aqueous supernatant was carefully transferred to a new microtube, and after adding 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volume of 100 % ethanol to the microtube, the mixture was incubated at -70 ℃ for 30 min. The precipitated DNA was centrifuged at 14,000 x g at 4 ℃ for 30 min. After discarding the supernatant, the pellet was washed with 70 % ethanol. The extracted DNA was collected after further centrifugation, and the final yield of DNA was dissolved in 50 ㎕ sterile deionized water. The concentration of genomic DNA was determined with Gene Quant RNA/DNA Caculator (Amersham Pharmacia Biotech. USA).

B. Lymphocyte DNA preparation

Peripheral blood lymphocytes from each patient and volunteers were collected in heparinized vacutainer tubes (Beckton Dickinson, Franklin Lakes, NJ). Lymphocytes were isolated using Ficoll-Pague (Pharmacia, Uppsala, Sweden) following the manufacturer's instructions. Genomic DNA from lymphocytes was isolated with the LaboPass™ Blood kit

(Genotein Biotech. Korea). The samples were washed in 1 X phosphate buffered saline (PBS) and centrifuged for 2 min at 14,000 x g. The pellet was resuspended in 200 ㎕ of 1 X PBS, 200 ㎕ of lysis buffer and 20 ㎕ of proteinase K (20 mg/ml). The mixtures were incubated for 10 min at 56 ℃, mixed with 200 ㎕ of 100 % ethanol and transferred to a spin column. These mixtures were centrifuged for 1 min at 14, 000 x g. Then, the spin column was washed with 700 ㎕ of Buffer BW (Genotein Biotech. Korea) and was centrifuged for 1 min at 14, 000 x g. The spin column was washed again with 500 ㎕ of Buffer NW (Genotein Biotech. Korea) and was centrifuged for 1 min at 14, 000 x g. DNA was eluted in sterile deionized water and stored at -70 ℃.

7. SNP target determinations

Target SNPs were determined using available SNP databases in public. GeneCards (http://bioinformatics.weizmann.ac.il/cards) and OMIM (http://www.ncbi.nlm.nih.gov/Omim) databases were utilized to gain

information about the genes in the gemcitabine metabolic pathway enzymes. Database queries for SNPs in the gemcitabine metabolic pathway were conducted in HOWDY (http://gdb.jst.go.jp/HOWDY/) which includes dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) and JSNP (http://snp.ims.utokyo.ac.jp/).[12]

8. PCR Reaction

All amplification reactions were performed in an Eppendorf Mastercycler Gradient (Brinkmann Instruments, Inc. USA). PCR was performed using template DNA (200 ng), dNTPs (2.5 mM), 10 X PCR buffer with $MgCl_2$ (1.5 mM) and *Taq* polymerase (5 U; Super Bio Co, Ltd. Korea) in total 50 ㎕ reaction volumes. The used primer sequences were as follows: DCTD exon 3 (F:5'-CATCAGCAATGAGCTACTGA, R:5'-TGCAACCAAAGTTTTTCTTT), dCK exon 4 (F:5'-CCACTGG ATTTAGGAGAATG, R:5'-GTGAAACACATTTTTATTGGG), dCK exon 5 (F:5'-AAAAGAAAATTTTGATGGCA, R:5'-ACTTCAGTGTCCTAT

GCAGG), RRM1 exon 9 (F:5’-TTGATTTTATTTGGGCATTT, R:5’-CAATTCATGGAGCATACCTT), RRM1 exon 19 (F:5’-TTCCTTGTAGG GTTTGAAGA, R:5’-AGGATCCACACATCAGACAT). The condition of PCR amplification were as follows: 95 ℃ for 5 min followed by 94 ℃ for 30 s, 57 ℃ for 20 s and 72 ℃ for 30 s, then 72 ℃ for 2 min, repeated by 30 cycles. All PCR products were purified using PCR-Clean up kit (GENENMED Inc. Korea). The DNA was eluted in sterile deionized water and stored at -70 ℃ before sequencing.

9. DNA sequencing

Direct sequencing of each PCR product was carried out using CEQ^{TM} 8000 genetic analysis system (Beckman Coulter, Inc. USA). Reaction mixture contained 25-100 fmol of purified PCR products, 1.6 pmol/㎕ of either the sense or antisense oligonucleotides (same as PCR primer) and DTCS premix (10 X sequencing buffer, dNTP mix, ddUTP / ddCTP / ddATP / ddGTP Dye terminator, polymerase enzyme). Each cycle of the

sequencing reaction consists of 96 ℃ for 20 s, 50 ℃ for 20 s and 60 ℃ for 4 min. To stop the sequencing reaction, a Stop solution/ glycogen mixture [2 ㎕ of 3 M sodium acetate (pH 5.2), 2 ㎕ of 100 mM Na_2-EDTA (pH 8.0) and 1 ㎕ of 20 mg/mL of glycogen] was used. And after addition of 60 ㎕ of 100 % ethanol, the mixture was centrifuged for 15 min at 14,000 x g. The supernatant was discarded and the pellet was washed with 70 % ethanol. The pellet was dissolved in 40 ㎕ of the Sample Loading Solution (CEQ Dye Terminator cycle sequencing kit, Beckman Coulter, Inc. USA.). The samples were transferred to a polypropylene sample plate and covered by mineral oil. The sample plate was then loaded on the CEQ^{TM} 8000 genetic analysis system and fluorescence was detected. Sequence variants were compared with reference sequences obtained from GenBank.

10. Real-Time PCR Assay

The cells were scraped from the plates, pelleted and washed three

times in 1 X Dulbecco's PBS (Invotrigen Corporation, USA). RNA extraction was performed using TRIzol Reagent (Invitrogen Corporation, USA) and RNA quantification was done using Gene Quant RNA/DNA Calculator (Amersham Pharmacia Biotech. USA). The RNA pellets were dissolved in nuclease-free water and stored at -70 ℃ prior to use. 2 ㎕ of cDNA of each sample was used for the real-time PCR assay. The total volume of the reaction mixture was 20 ㎕, which contained 10 ㎕ of QuantiTect SYBR Green PCR mixture including 2.5 mM $MgCl_2$ (QIAGEN, CA, USA), 2 ㎕ of the cDNA and 20 pmol of each primer (Proligo, Singapore) in distilled water. The used primer sequences were as follows: RRM1 (forward 5′-ATCAGGACTGGTCTTTGATG-3′, reverse 5′-TGAGACTCAATGATGGCATA-3'), β-actin (forward 5'-GGGAATTCAAAACT GGAACGGTGAAGG-3', reverse 5'-GGAA GCTTATCAAAGTCCTCGGCCACA-3'). PCR was performed at 95 ℃ for 15 min in order to activate the HotstarTaq DNA polymerase, and then for 35 cycles of amplification at; 95 ℃ for 20 s, 50 ℃ for 30 s,

72 ℃ for 45 s on a Rotor Gene 2072D real-time PCR machine (Corbett Research, Australia). The amplified fluorescence signal in each specimen was measured at the late extension step of each cycle. In order to quantify each gene, we used 10-fold serially diluted human genomic DNA (Promega, Madison, USA). The standard curve was drawn by plotting the measured threshold cycle versus the arbitrary unit of the copies per reaction based on the β -actin gene expression of serially diluted genomic DNA. The threshold cycle (C_t) values were determined as the cycle number at which the fluorescence exceeded the threshold value. In the negative control, there was no fluorescent signal when the cycle number was increased to 35.

11. Western Blotting

After harvesting, the cells were resuspended in 50 ㎕ of lysis buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and protease inhibitor mixture.] Cell nuclei and debris were pelleted by

spinning at 14,000 x g in a refrigerated tabletop microcentrifuge for 20 min. The aqueous supernatant was carefully transferred to a new microtube, and it was centrifuged for 10 min at 14, 000 x g. The samples were boiled to denature the proteins, and the protein amount was quantitated using the Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein (20 μg) were separated on a 10 % SDS- polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Immobilon-P transfer membrane, Millopore Corporation, USA) in 20 % methanol / 1 X Tris-glycine transfer buffer. Membranes were blocked overnight in 5 % skim milk (DIFCO®, USA) in 1 X TBS and 0.1 % Tween 20 (Amresco Inc, USA). Primary antibody incubations were done in blocking buffer for 2 hr at room temperature. Blots were washed several times with 1 X TBS and 0.1 % Tween 20 before secondary antibody exposure (1 hr at room temperature). Antibodies used were mouse monoclonal anti-RRM1 (1:500; Chemicon International, USA), anti-mouse IgG HPL whole antibody (1:2000;

Amersham Pharmacia Biotech, USA), anti-β-actin (Abcam Ltd. Cambridge, UK). After the washing, detection was done using the ECL Western blotting reagents (Amersham Pharmacia Biotech, USA). After adding the blotting reagents, the membrane was exposed to high performance autoradiography film (Amersham Pharmacia Biotech, Little Chalfont, UK).

12. Statistical analysis

Clinical data analysis was performed using the SPSS 10.0 program (SPSS, Chicago, IL, USA). Time-dependent variables were estimated with a Log rank test using the Kaplan-Meier method. Allele or genotype frequency differences between the patient and control populations were determined using a χ^2 - test. Significant differences from Hardy-Weinberg equilibrium were calculated using SNP analyzer software (http://www.istech21.com/bionics/consulting_6.htm). Haplotypes were constructed from genotype data using the EMHAPERE program.[29] For

the clinical correlation analysis, a logistic regression was used for the multivariate analysis of genotype and other clinically relevant variables. The results were considered as significant when bilateral p-values were < 0.05.

III. RESULTS

1. Characteristics of patients and healthy volunteers

Baseline characteristics for the seventy-four patients are shown in Table 1. Median age was 50 years (range, 31-70 years) and all tumors were infiltrative ductal carcinoma except two infiltrative lobular carcinomas. EOCG performance status was 0 to 2 in all patients. The fifty-six control samples were obtained from healthy volunteers, with the median age of 40 years (range, 20-64 years).

Table 1. Patient characteristics

Patients	Number (%)	Healthy volunteer	Number (%)
Total number	74		56
Evaluated patients	71		
Median age (years)	50		40
(range)	(31-70)		(20-64)
Performance status (%)			
0-1	46 (62.2)		
2	28 (37.8)		
Menopausal status (%)			
Premonopausal state	50 (67.6)		
Postmenopausal state	24 (32.4)		
Histology (%)			
Ductal carcinoma	72 (97.3)		
Lobular carcinoma	2 (2.7)		
Hormone receptor (%)			
ER[a] + PR[b] +	24 (32.4)		
ER + PR -	23 (31.1)		
ER - PR +	9 (12.2)		
ER - PR -	16 (21.6)		
Unknown	2 (2.7)		

a. ER: estrogen receptor, b. PR: progesterone receptor

2. Treatment response and toxicity

Among 71 evaluable patients, one complete response (1.4 %), 13 partial responses (18.3 %) and 26 stable diseases (36.6 %) were documented. Overall response rate was 19.7 % and the disease control rate was 56.3 %. All the patients were evaluable for toxicity. The most common toxicity was neutropenia, and 32.4 % of patients reported grade III-IV neutropenia. Non-hematologic toxicity was mild and only grade II diarrhea was reported in 12.2 % (Table 2).

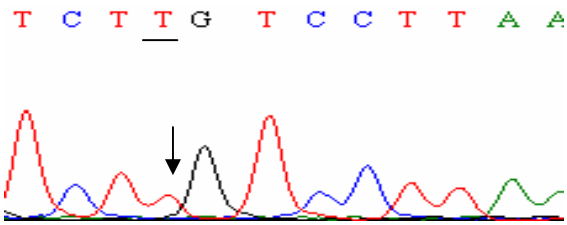
Table 2. Toxicity evaluation of chemotherapy

Toxicity	WHO grade				
	0 (%)	I (%)	II (%)	III (%)	IV (%)
Hematologic toxicity					
Leucopenia	11 (14.9)	19 (25.7)	28 (37.8)	16 (21.6)	0 (0.0)
Neutropenia	14 (18.9)	15 (20.3)	21 (28.4)	22 (29.7)	2 (2.7)
Anemia	1 (1.4)	29 (39.2)	38 (51.4)	6 (8.1)	0 (0.0)
Thrombocytopenia	40 (54.1)	14 (18.9)	16 (21.6)	3 (4.1)	1 (1.4)
Non-hematologic toxicity					
Diarrhea	21 (28.4)	44 (59.5)	9 (12.2)	0 (0.0)	0 (0.0)

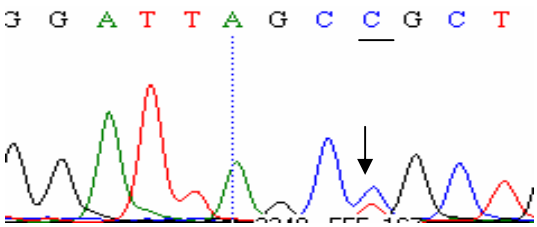
3. Frequencies of SNP genotypes

We investigated the 7 SNP sites of gemcitabine metabolism-related genes of DCTD (2 sites), dCK (2 sites) and RRM1 (3 sites) (Figure 1). The incidences of SNPs in breast cancer patients were 1.4 % of dCK (626 A>G), 10.8 % of DCTD (315 T>C), 40.5 % of the 1st RRM1 (2455 A>G), 44.6 % of the 2nd RRM1 (2464 G>A) and 23 % of the both RRM1 sites (Table 3). In healthy volunteers, the SNPs were not found in dCK, 7.1 % of DCTD, 50.0 % of the 1st RRM1, 64.3 % of the 2nd RRM1 and 53.6 % of the both RRM1 sites. When the genotype and allele distribution of these 3 genes were evaluated by Hardy-Weinberg equation, there were no differences between control group and breast cancer patients in SNPs frequencies (data not shown).

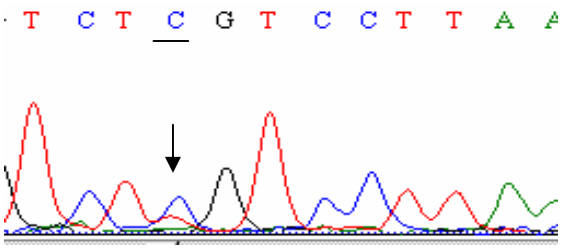
A. T2455T WT (anti-sense)



B. A2464A WT (anti-sense)



C. T2455T A>G (anti-sense)



D. A2464A G >A (anti-sense)

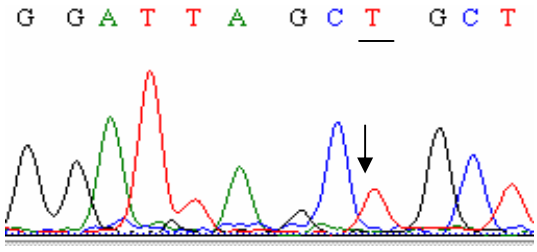


Figure 1. Electropherogram of 2 non-synonymous SNPs and their flanking sequences in RRM1. The electropherograms are shown in reverse orientation as indicated. Arrows indicate the polymorphic and homozygous positions and the homozygous nucleotides are underlined. (A), (C) Two alleles (A and G) with an adenine or guanine 2455 nucleotides of the RRM1 site were observed giving rise to the polymorphism RRM1 2455 A>G. (B), (D) Two alleles (C and T) with an cytosine or thymine 2464 nucleotides of the RRM1 site were observed giving rise to the polymorphism RRM1 2464 G>A.

Table 3. Frequencies of each SNP in breast cancer patients and normal control group

Gene	SNP site	Genotype	Amino acid change	Patients (n=74)		Normal control (n=56)	
				Wild type (%)	Homozygous (%)	Wild type (%)	Homozygous (%)
DCTD[a]	Exon4	255 G>C	Ala/Ala	74 (100)	0 (0.0)	56 (100)	0 (0.0)
		315 T>C	Val/Val	66 (89.1)	8 (10.8)	52 (92.5)	4 (7.1)
dCK[b]	Exon4	626 A>G	Gln/Arg	73 (98.6)	1 (1.4)	56 (100)	0 (0.0)
	Exon5	753 A>G	Gln/Gln	74 (100)	0 (0.0)	56 (100)	0 (0.0)
RRM1[c]	Exon9	1082 C>A	Arg/Arg	65 (87.7)	9 (12.1)	28 (50.0)	28 (50.0)
	Exon19	2455 A>G	Thr/Thr	44 (59.5)	30 (40.5)	17 (30.4)	39 (69.6)
		2464 G>A	Ala/Ala	41 (55.4)	33 (44.6)	20 (35.7)	36 (64.3)
				57 (77.0)	17 (23.0)	26 (46.4)	30 (53.6)

a; deoxycytidine monophosphate deaminase, b; deoxycytidine kinase, c; ribonucleotide reductase M1 polypeptide.

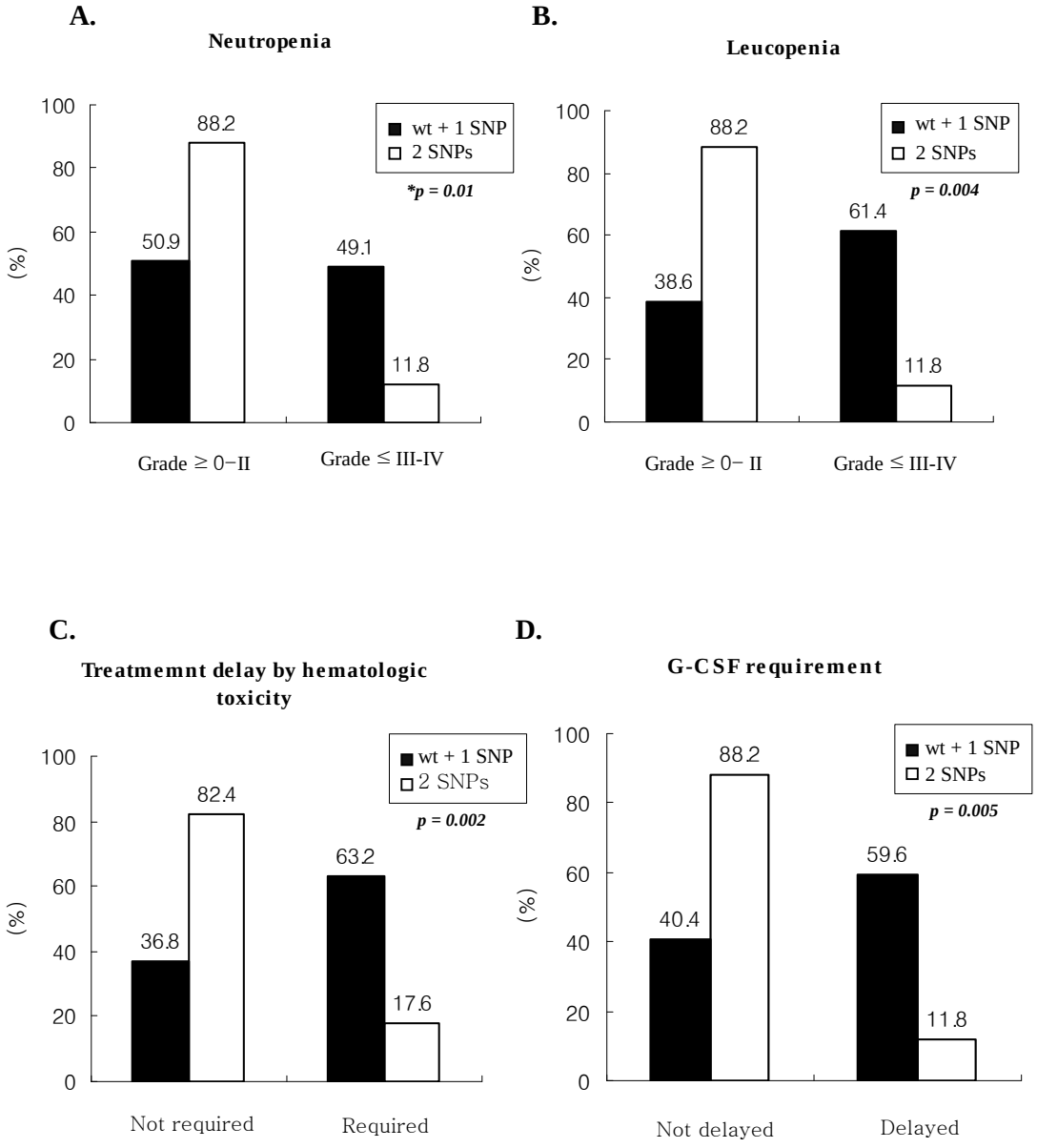
4. Correlation of RRM1 genotype and treatment toxicity or response

Based on the RRM1 genotypes of breast cancer patients, the correlation between genotype and the treatment toxicity was analyzed. The pattern and grade of toxicity of the group having any type of one polymorphism were similar to those of wild type group. But less toxicities such as neutropenia ($p < 0.01$), leucopenia ($p < 0.004$), treatment delay by hematologic toxicity ($p < 0.002$), and G-CSF requirement ($p < 0.004$) (Figure 2) were observed in patients with RRM1 double polymorphisms. We also evaluated the association between genotypes and treatment response. However, we could not observe any significant correlation between genotype and tumor response (data not shown).

5. Correlation of RRM1 haplotype and toxicity, response or survival

With 3 RRM1 genotypes, 6 haplotypes were found (Table 4). We analyzed the correlation between each haplotype and toxicity

parameters in breast cancer patients as we did with genotypes (Figure 2E). Strong correlation with neutropenia was also found when breast cancer patients had double polymorphism (2455 A>G plus 2464 G>A). Neither association with clinical response was observed with RRM1 haplotypes (data not shown). When we compared the disease-free survivals according to RRM1 haplotype, there was a tendency toward a poor survival with having more than double polymorphisms (Figure 4).


A.
Neutropenia
100
80
60
40
20
0
(%)
88.2
50.9
49.1
11.8
wt + 1 SNP
2 SNPs
*p = 0.01
Grade ≥ 0−II
Grade ≤ III-IV
B.
Leucopenia
88.2
38.6
61.4
11.8
wt + 1 SNP
2 SNPs
p = 0.004
Grade ≥ 0− II
Grade ≤ III-IV
C.
Treatmemnt delay by hematologic toxicity
82.4
36.8
63.2
17.6
wt + 1 SNP
2 SNPs
p = 0.002
Not required
Required
D.
G-CSF requirement
88.2
40.4
59.6
11.8
wt + 1 SNP
2 SNPs
p = 0.005
Not delayed
Delayed

E.

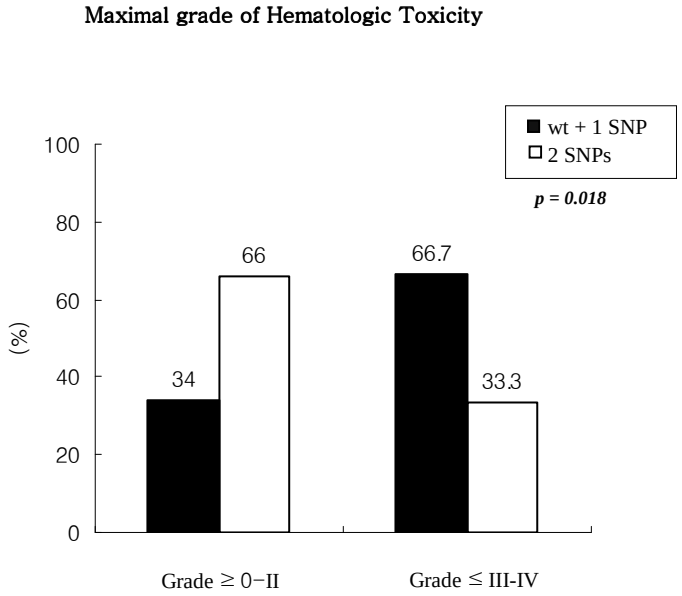


Figure 2. Incidence of gemcitabine toxicity based on RRM1 polymorphisms. Breast cancer patients were divided to two groups. 1st group composed of homozygous for wild type allele or have one variant allele. 2nd group composed of two variant alleles for the double polymorphisms of RRM1 (2455 A>G and 2464 G>A). (A) Neutropenia (B) Leucopenia (C) Treatment delay by hematologic toxicity (D) G-CSF requirement (E) Haplotype of RRM1 was related with gemcitabine toxicity in breast cancer patients. * was calculated with χ^2 test.

Table 4. Haplotypes of RRM1

	Haplotype*	Frequency (%)		A/G + G/A	
wt	C A G	26	(35.1)	0	(0.0)
1 SNP[a]	C A A	14	(18.9)	0	(0.0)
	C G G	10	(13.5)	0	(0.0)
	A A G	3	(4.1)	0	(0.0)
2 SNP[b]	C G A	16	(21.6)	16	(76.2)
	A G A	2	(2.7)	2	(9.5)
	A G G	3	(4.1)	3	(14.3)
	Total	74		21	

[a]1 SNP site; (2455 A>G or 2464 G>A), [b]2 SNP site; both site of RRM1 SNPs (2455 A>G plus 2464 G>A)

*Haplotypes were constructed from genotype data using EMHAPERE program.

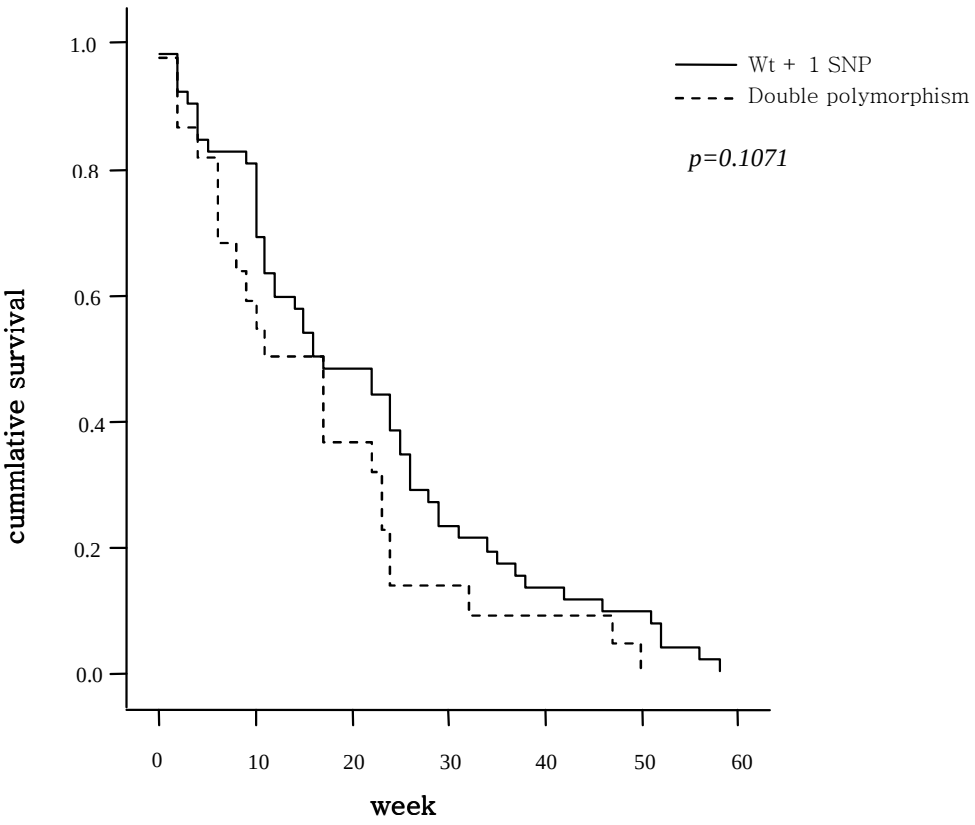


Figure 3. Progression-free survival according to haplotype of RRM1.

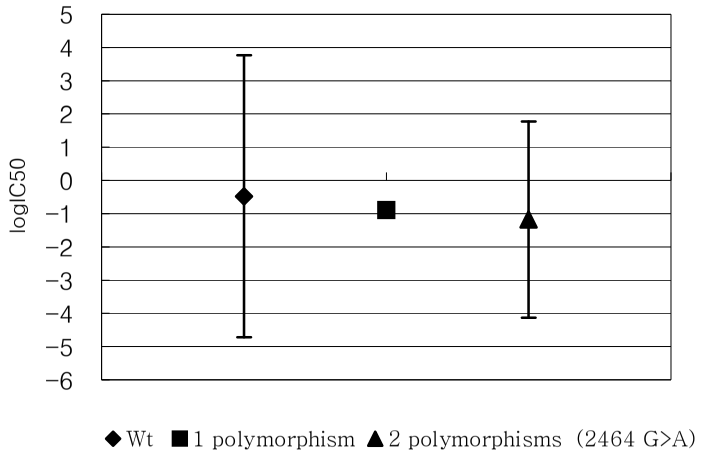
6. *In vitro* analysis with 10 breast cancer cell lines

We selected 10 breast cancer cell lines to perform association study was RRM1 genotypes with *in vitro* cytotoxicity to gemcitabine. Polymorphism of the RRM1 appeared more frequently in the cell lines also compared to dCK and DCTD. We observed that 8 cell lines with RRM1 variation (2464 G>A) showed increased sensitivity to gemcitabine (2.3 - fold) (Figure 4A).

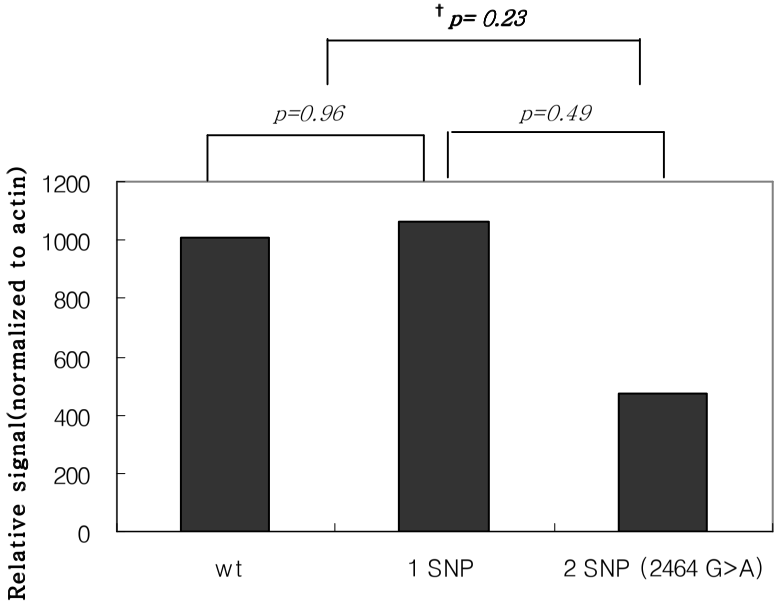
7. RRM1 expression in mRNA and protein

We also determined the RRM1 mRNA levels in each genotype pattern; we observed that 8 cell lines with RRM1 variation (2464 G>A) showed decreased mRNA level (Figure 4B). Western blot analysis confirmed the mRNA expression results. The level of RRM1 protein decreased in cell lines with RRM1 variation (Figure 4C).

A.


5
4
3
2
1
0
−1
−2
−3
−4
−5
−6
logIC50
◆Wt ■1 polymorphism ▲2 polymorphisms (2464 G>A)

B.


† p= 0.23
p=0.96
p=0.49
Relative signal(normalized to actin)
1200
1000
800
600
400
200
0
wt
1 SNP
2 SNP (2464 G>A)

C.

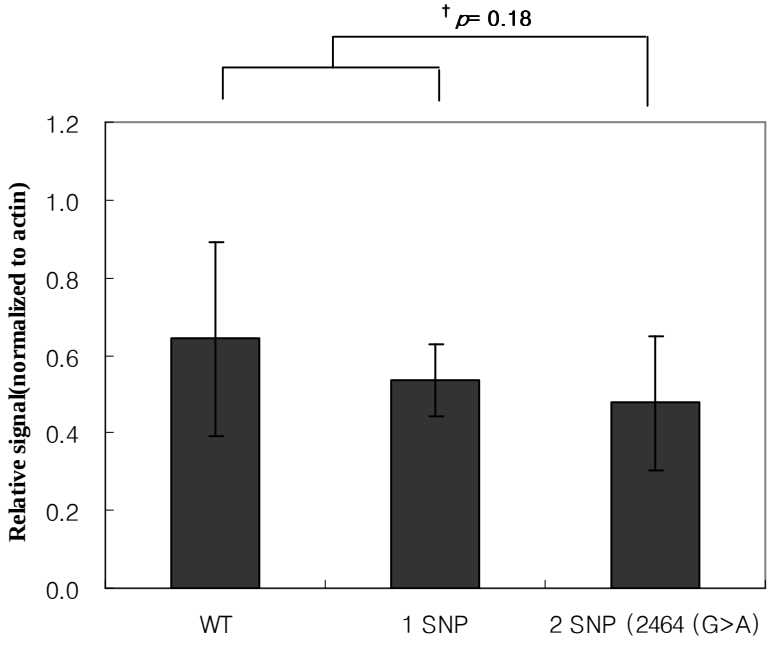


Figure 4. Response according to RRM1 genotypes in breast cancer cell lines. (A) Correlation of RRM1 genotype and cytotoxicity in breast cancer cell line. (B) Correlation of RRM1 genotype and mRNA expression in breast cancer cell line. (C) Western blot analysis of RRM1 protein. 20 μ g of cell extract protein was loaded in each lane. Protein extracts examined by Western blotting confirmed the PCR results. The cell line with RRM1 variation extract shown here was obtained from cells cultured three times in the absence of gemcitabine. Elevated RRM1 protein levels were also observed in cell line with RRM1 variation extracts compared with other samples. *wt: wild type allele.† was calculated with Student's t-test.

IV. DISCUSSION

Pharmacogenomic studies are rapidly elucidating the inherited nature of differences in drug disposition and effects, thereby enhancing drug discovery and providing a stronger scientific basis for optimizing drug therapy based on each patient's genetic constitution.[1-3]

Especially, some of these SNPs have been linked to interindividual differences in their effect on anticancer agents and toxicity of many medications. [4] Drug-metabolizing enzymes interact with transporters, receptors or other drug targets resulting in different responses or toxicity of the drug within patients with the same SNPs.[5] Our study aimed to evaluate the SNPs of gemcitabine metabolic pathway and genes as a predictive marker of clinical outcome. SNPs of metabolic genes or target molecules of gemcitabine were analyzed and compared with the drug metabolism.

We analyzed SNPs of gemcitabine related metabolite enzyme such as

DCTD, dCK and RRM1.[11] Gemcitabine must be activated through phosphorylation by the enzyme dCK to produce an active metabolite. The drug is inactivated by DCTD, which reduces therapeutic activity. RRM1 is involved in DNA synthesis and repair.[12] RRM1 has a 9 cSNP on chromosome 11. We selected three SNPs that are nonsynonmous SNP with no amino acid change and no splice variants. Also, RRM1 was distributed within the alpha subunit involved in nucleotide transport and metabolism. In the genotype analysis of each gene in breast cancer patients, DCTD and dCK showed genotype frequency lower than 10 %. However, the frequency of RRM1 single SNP such as 2455 A>G, 2464 G>A were above 40 %, 23 % in two SNPs. The same results were obtained with a control group. We compared these genotypes with the clinical outcome of each patient. Correlation with toxicity was significant when RRM1 had more than two SNPs. However, drug response showed no significant correlation with SNP. The same results were obtained in haplotype analysis. Patients with haplotypes of more than two SNPs

showed lower toxicity level. In the survival analysis of RRM1, patients having more than two SNPs showed shorter survival time than other patients. Based on these results, we suggest that patients with two SNPs (2455 A>G, 2464 G>A) are more resistant to gemcitabine than others genes such as DCTD and dCK. Our results demonstrate that the two SNPs in the RRM1 gene provide a more effective prediction on the clinical outcome of gemcitabine chemotherapy.

8 of 10 breast cancer cell lines having RRM1 variation (2464 G>A) showed increased drug sensitivity than other cell lines in the *in vitro* study. We compared the RRM1 genotype and mRNA expression level. It has been reported that the increase in mRNA level results in drug resistance; decrease in mRNA level results in drug sensitivity.[21] We confirmed these results in our study. However, in our study with cell lines with RRM1 variation, we observed increased drug resistance in spite of decreased mRNA level. The same result was obtained in breast cancer patients. Patients having two SNPs showed lower toxicity level,

suggesting that they are resistant to gemcitabine. With contradictoring data to previously reported results, we suppose that some unknown molecule or mechanism might be involved in gemcitabine metabolism. Although we cannot evaluate drug toxicity or efficacy with the SNPs of RRM1 only, these results demonstrate that the two SNPs (2455 A>G, 2464 G>A) have high correlation with drug toxicity, making SNPs a potential predictive marker.

V. CONCLUSION

In conclusion, in heavily pretreated breast cancer patients, gemcitabine monotherapy could be carried out effectively as salvage treatment by prediction of safety and dose adjustment on the basis of pharmacogenomic association study.

RRM1 genotyping in PBMC might be the useful biomarker to predict the treatment toxicity in gemcitabine monotherapy in breast cancer patients.

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ABSTRACT (IN KOREAN)

유방암 치료에서 gemcitabine 에 대한 치료효과와 부작용을 예측할 수 있는 유전자형 발굴

<지도교수 정현철>

연세대학교 대학원 의학과

최 연 호

Gemcitabine의 대사 과정에 관여하는 유전자의 단일염기다형성 (SNP: Single Nucleotide Polymorphism)을 통하여 유방암 환자들의 약제에 대한 부작용과 치료 효과를 예측할 수 있는 예측 인자를 선별하고자 하였다. 약제의 대사에 관여하는 deoxycytidine kinase (dCK), deoxycytidine monophosphate deaminase (DCTD), 그리고 약제의 대상인 ribonucleotide

reductase M1 polypeptide (RRM1) 의 3 가지 유전자를 선정하였다. Gemcitabine을 투여한 76 명의 진행성 유방암 환자들과 정상인 56명의 말초혈액, 그리고 10 개의 유방암 세포주로부터 추출한 genomic DNA를 이용하여 단일염기다형성을 분석하였다. 유방암 환자들의 각 유전자들의 SNP 빈도수는 1.4% (626 A>G), DCTD 10.8% (315 T>C), 1^{ST} RRM1 40.5% (2455 A>G), 2^{ND} RRM1 44.6% (2464 G>A), 그리고 RRM1의 2 부위 동시에 존재하는 경우가 23%로 나타났다. 이러한 결과는 정상군에서도 비슷한 결과를 나타내었다. 본 연구에서 확인한 RRM1의 2 부위 동시에 SNP가 존재하는 것(2455 A>G, 2464 G>A)은 알려져 있지 않는 결과였다. RRM1의 2 부위 동시에 SNP가 존재하는 경우를 토대로 하여 toxicity 지표인 neutropenia ($p < 0.01$), G-CSF requirement ($p < 0.005$) 비교 분석해 본 결과 높은 상관관계를 가지는 것을 관찰할 수 있었다. 또한 RRM1의 3가지 genotype을 이용하여 Hardy-Weinberg equation을 통해 haplotype을 구해 본 결과 앞선 genotype과 같은 결과를 얻을 수 있었다. 세포주를 이용한 *in vitro* 연구를 통해 SNP를 가지는 RRM1의 gemcitabine 약제 감수성과의 상관관계를 살펴보았다. 먼저 10

개의 유방암 세포주들의 약제에 대한 세포 독성 정도를 알아보기 위하여 MTT assay를 수행한 후 구해진 IC_{50} 값을 토대로 SNP 분석결과와 비교해 보았다. 그 결과 RRM1의 변이(2464 G>A)를 가지는 8 개의 세포주에서 gemcitabine에 대한 감수성이 증가한 것을 관찰할 수가 있었다. 또한 RT-PCR 과 western blot 을 통하여 RRM1의 mRNA와 단백질 발현 정도를 살펴 본 결과 RRM1의 유전적 변이를 가지는 8개의 세포주에서 mRNA와 단백질 발현 정도가 변이를 가지지 않는 세포주에 비해서 감소하는 경향을 관찰할 수 있었다.

결론적으로 본 연구에서는 gemcitabine 단독 치료를 수행한 유방암 환자의 말초혈액 단핵구세포(PBMC)에서 추출한 genomic DNA를 이용하여 RRM1 유전자를 약제에 대한 부작용과 치료효과를 예측할 수 있는 생물학적 표지자로 사용될 수 있을 것으로 기대된다.

핵심되는 말: Gemcitabine, 단일염기다형성 (SNPs : Single nucleotide polymorphism), DCTD (deoxycytidine monophosphate deaminase), dCK (deoxycytidine kinase), RRM1 (ribonucleotide reductase M1 polypeptide), 약물유전체학 (pharmacogenomics)