STUDY THE ANTICANCER MECHANISM OF THE PROMISSING COMPOUND 2B2D BY USING MICROARRAY TECHNIQUE

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Received: 18 November 2011; Accepted for publication: 17 November 2012

ABSTRACT

Being a modern technique with the ability of studying, discovering, probing and analyzing the expression of thousands genes, even the whole genome in the only one experiment, microarray proved to be a powerful tool for cancer research especially at molecular level. Employing the potential anticancer compound 1-(5,7-dimetoxy-2,2-dimetyl-2H-cromen-8-yl)-but-2-en-1-on (2B2D in short) and LU-1, the human lung cancer cells as research objects, we successfully hybridized the cy3/5 incorporated cDNA with Phalanx HOV5 microarray. The results showed 742 genes that got effected with equal or over two folds change under 2B2D treatment. Among - those, 386 genes were up-regulated while the other 356 were down-regulated. The *Nuclear factor (erythroid-derived 2, regulatory factor X domain containing 1, fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism)* and *E2F transcription factor 8* genes were the most stimulated by our compound. The genes that named as *Solute carrier family 7 (cationic amino acid transporter, system) member 11, kelch-like 24* and *Hypothetical LOC344887* were the most down in action.

Keywords: cDNA, cyanine, LU-1, Mallotus apelta, microarray, spectrophotometer

1. INTRODUCTION

The compound 1-(5,7-dimetoxy-2,2-dimetyl-2H-cromen-8-yl)-but-2-en-1-on (2B2D in short) found in the Vietnamese plant *Mallotus apelta* was patent registered by The Institute of Natural Product Chemistry, Vietnam Academy of Science and Technology for its novel chemical structure and the anticancer effect as well. The compound was proved to possess the effective anticancer ability *in vitro*. The pharmaceutical activity of this promising compound is in the process of *in vivo* testing [1]. However, the molecular acting mechanism of this compound had not been studied. In order to study the working mechanism of a promising agent, microarray technique has been recently proved to be effective and feasible.

Based on Southern Blotting technique, microarray is learned as a new tool for gene expression analysis. Microarray is the amazing thing in science following the availability of

human genome sequence information which allow us to do experiments on a genome-wide scale. DNA microarrays offer the ability to look at the expression of thousands of genes or even the whole enome of a living species in a single experiment [2, 3, 4]. This technology provides a versatile plattform for utilizing information from the Human Genome Project to benefit human health, especially in cancer research [5].

A microarray is an orderly arrangement of known or unknown DNA samples attached to a solid support. Each DNA spot on the microarray (called the probe) is usually less than 200 micrometer in diameter and an entire array typically contains thousands of spots. Many different design formats are possible. The probes attached to the solid support can be small oligonucleotides, cDNAs or genomic sequences [6]. The array may be formatted by photolithographic synthesis of oligonucleotides *in situ*, or the DNA samples may be applied directly to the array surface by pins, nibs or inkjet technology [7].

Basically, measuring the mRNA level will create the profile of gene expression of under study sample at the testing time. Expression profiling is very important because it is the set of expressed genes and interplay between the products encoded by them that determines the phenotype of a cell [8]. The microarray experiment involves in preparations the fluorescently labeled cDNA from mRNA, isolated from two different conditions, to be compared from two contrasted colors such as cy3 (green) and cy5 (red). The resulting mixture of labeled cDNAs is hybridized to a large number of genes placed individually spotted on a microarray glass slide. Hybridization results are then be analyzed by determining the relative fluorescent intensity at each gene spot with the use of a laser scanner. Spots that fluoresce dominantly with one label or the other indicate a gene that differentially up-regulated or down-regulated in the sample under the condition of the study [9].

(A) (B)

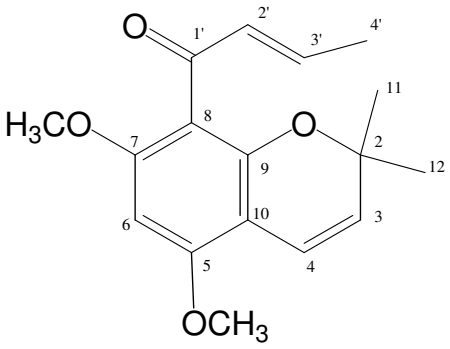

Figure 1. (A) Chemical structure of the compound 2B2D; (B) The image of the box containing a whole human genome microarray (from Phalanx)

Learning all advantages of microarray, we decided to apply this technique for studying the anticancer mechanism of the compound 2B2D at molecular level. The results of our research are announced in the below report.

2. MATERIALS AND RESEARCH METHODS

2.1. Materials

The compound 2B2D was kindly provided by Assoc. Prof. Phan Van Kiem, Institute of Natural Product Chemistry (INPC), Vietnam Academy of Science and Technology (VAST). The whole human genome microarrays were purchased from Phalanx Biotech Group. The other chemicals such as RNase-free water (DEPC treated), oligo dT primer (16- to 18-mer) at 1 µg/µl, 3 M Sodium Acetate, pH 5.2, 100% Ethanol, 70 % Ethanol, Superscript II Reverse Transcriptase, 5X first strand buffer RNase A (4 mg/ml), RNase H (2 unit/µl), PCR CleanUp Kit, RNeasy® RNA Isolation kit etc. were purchase from Qiagen, Invitrogen etc.

The cell line LU-1 (human lung carcinoma) was kindly provided by Prof. Dr. J M Pezzuto, University of Hawaii at Hilo, US.

2.2. Methods

LU-1 cells treatment with 2B2D for total RNA extraction: The cells LU-1 were cultured in the DMEM (GIBCO) supplemented with 10 % FBS, 1 % PSF, 10 % Sodium pyruvat (GIBCO). The cells were subcultured after 3 - 5 days at the rate (1 : 3) and kept in the incubator at 37 ^{o}C, 5 % CO_2. LU-1 were seeded at the concentration 1×10^6 cells/dish and treated with 2B2D at 0,5 µg/ml and 1 µg/ml. DMSO 0.1 % was used as control.

Total RNA isolation and purification: After 24 hours treated with 2B2D, the cells LU-1 were collected for total RNA isolation with the use of RNeasy® RNA Isolation kit (Qiagen). The purified RNA were quantitied by using Nanodrop ND-1000 version 3.3.0 Spectrophotometer (Thermo Scientific) and qualified by electrophoresis on agarose gel.

cDNA synthesis from total RNA: Since this process involves in fluorescence, all research steps require the dark condition strictly. Toward 10 - 20 µg total RNA or 0.1 - 0.5 µg of tRNA, the contents of one reaction will be: 8 µL 5X First Strand reaction buffer (Superscript II, Invitrogen); 1,5 µL AncT primer (5'-T20VN, 100 pmol/µL); 3 µL dNTP-dCTP (6.67 mM dATP, dGTP, dTTP, GE Healthcare); 1 µL 2 mM dCTP (GE Healthcare); 1 µL 1 mM Cyanine 3-dUTP or Cyanine 5-dUTP (GE Healthcare); 4 µL 0.1 M DTT (Invitrogen); 20 µg total RNA; Nuclease-free water up to 40 µL. Incubation of the reaction at 65 ^{o}C in 5 minutes in the dark to degrade the total RNA. Cooling the samples at 42 ^{o}C in 5 minutes before adding enzyme. Providing 2 µL reverse transcriptase (SuperScript II, Invitrogen) and continuing the reaction at 42 ^{o}C in 2 hours. Stop the reaction by adding 4 µL 50 mM EDTA (pH 8.0) and 2 µL 10 N NaOH; Neutralization by adding 4 µL 5 M acetic acid. Clean up the CyDye-cDNA with Qiagen PCR CleanUp Kit.

Microarray Hybridization: The whole process with pre-hybridization, hybridization and washing steps were completed by following microarray producer's instructions with some minor modification to fit our laboratory conditions.

Scanning, Imaging and Data analysis: The **GeneTAC UC-4** system of GENOMIC SOLUTIONS was used to scan the array after hybridization. The image were captured by this system by followings the instruction of the machine producer. And the raw data as well as the images were sent to PHALANX BIOTECH. GROUP, Taiwan for data analysis.

3. RESULTS AND DISCUSSION

There are three major steps involving to a typical microarray experiment: preparation of the array; preparation of Cdna probes and hybridization; and final scanning, imaging and data analysis. We hereby reported our experiment results with the Human OneArray® Whole Genome DNA V5 microarray.

3.1. Array preparation

Human OneArray® Whole Genome DNA V5 microarrays are made of sense-strand polynucleotide probes spotted onto a proprietary chemical layer coated on top of a 1" × 3" (25 mm × 75 mm) standard-format microarray glass slide. Updated information of genome content from public domains is used to design approximately 30,000 highly sensitive long-oligonucleotide probes for monitoring the expression level of corresponding protein-coding genes. Each probe is spotted onto the array in a highly consistent manner using a proprietary, non-contact spotting technology adapted for microarray manufacturing. Each microarray contains 30,275 oligonucleotides: *29,187 human genome probes*, and *1,088 experimental control probes*. Each oligonucleotide probe is designed to hybridize to a specific target gene described in the current public domain contents, such as NCBI Reference Sequence (RefSeq) Database and Ensembl Database.

According to the producer's instruction, the array requires a pre-hybridization step prior to hybridization of the labeled target. The pre-hybridization step reduces background signals and increases the performance of the microarray. The pre-hybridization step was completed by carefully following the instructions of the producer. After pre-preparation, the array could be kept in the dark box for as long as one week before cDNA hybridization.

3.2. Results of cDNA probing and Hybridization

As reported in another paper, the optimized conditions for 2B2D treatment on LU-1 cells were at 0,5 µg/ml and 1 µg/ml in the period of 24 hours and 48 hours of experiment in order to receive the maximum amount of tRNA. However, 0.5 µg/ml of 2B2D treated at 24 hour condition were decided for further microarray study.

Good dye incorporation rates are important for yielding the best data from microarray hybridization. Incorporation rates of 30-60 dye molecules per 1000 bases (17 - 33 bases /dye molecule) yield the most usable data. Rates below 20 dyes per 1000 bases (50 bases / dye) are very low and may lead to loss of signal of many targets. It is not recommended to perform hybridization with samples of low dye incorporation efficiency.

After fluorescent probing, the cDNA samples were quality checked by electrophoresis on 1 % (w/v) TAE agarose gel. The figure 3 proved that fluorescent dye cyanine was firmly incorporated into cDNA molecules. The cDNA samples therefore were ready for hybridizing with the microarray. We also calculated the pmol of cDNA samples that probed with cyanine by using the OD values from UV-visible spectrophotometer. All tested samples had over 142 pmol of cDNA labeled with probes (data not showed). Thus, the cDNAs were qualified for hybridization with the Human OneArray® Whole Genome DNA V5 microarray.

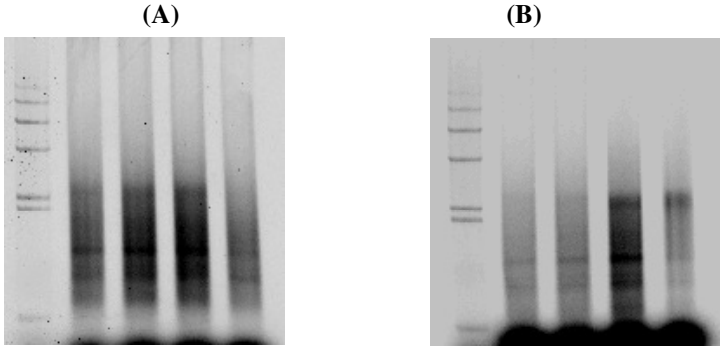

Figure 2. (A) The 2B2B treated sample probing with Cy5; (B) The control sample probing with Cy3

After following the instruction of Phalanx Biotech to hybrid our cDNA samples with the pretreated array, the slide were transferred to GENOMIC SOLUTIONS scan system in order to get the array image. The scanned array was showed in the figure 3.

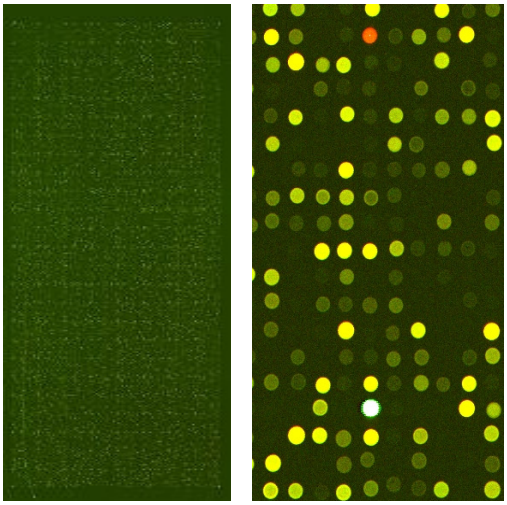

Figure 3. Images of array after hybridizing with Cy3/5 labeled cDNAs

The images displayed that labeled cDNA samples were hybridized well with the probes on the array. The color of the gene spots were totally clear. The image was also very sharp showing the success of the hybridization. There was big different in colors of probes. The red color showed that genes were up-regulated under 2B2D treatment while the green color implied down-regulated genes. The most popular color was yellow proved that most of genes were not clearly effected by 2B2D exposure. However, the image and the scanning software of GeneTAC4 only provided several basic information. To achieve the up or down-regulated gene list, we needed to analyze the data with GeneSpring software from Agilent.

3.3. Data analyzing results

After sending the raw data to Phanlanx Biotech. Group for analysis, we achieved the whole list of up and down-regulated genes of LU-1 cells under 2B2D treatment.

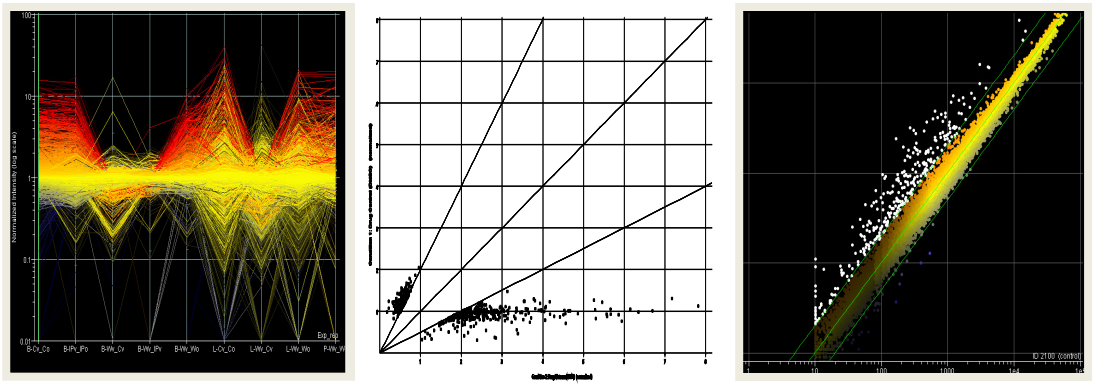

Figure 4. Data analysis with GENESPRING GX*7.3 software (Agilent Biotechnology)

By using GENESPRING GX*7.3 software (Agilent Biotechnology) to analysis the data, we found out 742 genes that got equal or over two fold changes. Among of those, there were 386 genes up-regulated while the other 356 genes down-regulated. The Table 1 showed all details of the 5 genes that were the strongest effect by the drug treatment either up or down-regulation.

Table 1. The list of the 10 genes with highest fold changes

Up-regulated	**fold change**	**Genbank**	**Gene Symbol**	**Description**
209930_s_at	5.985	L13974	NFE2	nuclear factor (erythroid-derived 2), 45kDa
237086_at	5.983	AI693336		
1552673_at	5.159	NM_173560	RFXDC1	regulatory factor X domain containing 1
204380_s_at	3.978	M58051	FGFR3	fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism)
219990_at	3.743	NM_024680	E2F8	E2F transcription factor 8
Down-regulated	**fold change**	**Genbank**	**Gene Symbol**	**Description**
207528_s_at	7.316	NM_014331	SLC7A11	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11
209921_at	7.405	AB040875	SLC7A11	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11
226158_at	7.583	AL120021	KLHL24	kelch-like 24 (Drosophila)
241418_at	7.725	AI819386		Hypothetical LOC344887
217678_at	7.728	AA488687	SLC7A11	solute carrier family 7, (cationic amino acid transporter, y+ system) member 11

From the list above we can see the nuclear factor (erythroid-derived 2, regulatory factor X domain containing 1, fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism) and E2F transcription factor 8 were up-regulated. Those genes were stimulated by our compound 2B2D. The other genes that encoded for solute carrier family 7 (cationic amino acid transporter, y+ system) member 11, kelch-like 24 and Hypothetical LOC344887 were strongly inhibited lead to down in action.

4. CONCLUSION

By using the advantages of microarray technique, we investigated the acting mechanism of the potential anticancer compound 2B2D at the molecular level on LU-1 cells. There were 386 genes up-regulated while the other 356 genes down-regulated. The nuclear factor (erythroid-derived 2, regulatory factor X domain containing 1, fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism) and E2F transcription factor 8 were the most

stimulated by our compound. The genes that named as solute carrier family 7 (cationic amino acid transporter, y+ system) member 11, kelch-like 24 and Hypothetical LOC344887 were the most down in action.

Acknowledgment. We thank Prof. Dr. John M Pezzuto, University of Hawaii at Hilo for kindly providing LU-1 cells. We also would like to thank Dr. Phan V Kiem, Institute of Marine Biotechnology, VAST, for providing the compound 2B2D.

This work was supported by the grant No.CS09_2 awarded by the Institute of Biotechnology, Vietnam Academy of Science and Technology.

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TÓM TẮT

NGHIÊN CỨU CƠ CHẾ KHÁNG UNG THƯ CỦA HỢP CHẤT TIỀM NĂNG 2B2D BẰNG KĨ THUẬT MICROARRAY

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Là một kĩ thuật mới với khả năng nghiên cứu, phát hiện, giám sát và phân tích sự biểu hiện của hàng ngàn, thậm chí là cả hệ genome trong cùng một thí nghiệm duy nhất, microarray tỏ ra là một công cụ hữu hiệu giúp ích cho các nghiên cứu về ung thư học, đặc biệt là về mặt cơ chế phân tử. Với việc sử dụng hoạt chất tiềm năng chống ung thư 1-(5,7-dimetoxy-2,2-dimetyl-2H-cromen-8-yl)-but-2-en-1-on (gọi tắt là 2B2D) và dòng tế bào ung thư phổi người LU-1 làm đối tượng nghiên cứu, chúng tôi đã lai thành công các mẫu cDNA có gắn chất nhuộm màu huỳnh quang cyanine 3/5 với chip Phalanx HOV5 microarray. Kết quả cho thấy 742 gen có mức độ biểu hiện bị thay đổi lớn hơn hoặc bằng hai lần khi chịu tác động của hoạt chất 2B2D. Trong số này, 386 gen tăng cường hoạt động và ngược lại, có 356 gen bị giảm mức độ biểu hiện. Các gen qui định cho *Nuclear factor (erythroid-derived 2, regulatory factor X domain containing 1, fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism)* và *E2F transcription factor 8* bị kích thích và có mức độ biểu hiện tăng cường mạnh nhất dưới tác động của hoạt chất. Các gen có tên như *Carrier family 7 (cationic amino acid transporter, y+ system) member 11, kelch-like 24* và *Hypothetical LOC344887* bị ức chế biểu hiện mạnh nhất.

Từ khoá: cDNA, cyanine, LU-1, Mallotus apelta, microarray, spectrophotometer