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Effects of aspirin on metastasis-associated gene expression detected by cDNA microarray[1]

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KEY WORDS aspirin; ovarian neoplasms; gene expression; neoplasm metastasis; cDNA microarray

ABSTRACT

AIM: To investigate the effect of aspirin on the metastasis-associated gene expression in 3AO ovarian cancer cells. **METHODS**: 3AO cells were treated with aspirin at the concentration of 1.2 mmol/L for 16 and 48 h, respectively. The total RNA was extracted with Trizol reagents and reverse transcribed with Superscript II and hybridized with cDNA microarray (containing oncogenes, tumor suppressor genes, signal transduction pathway molecules , adhesive molecules, growth factors and ESTs) fabricated in our lab. After normalization, the ratio of gene expression of aspirin treated to untreated 3AO cells being either 2 fold up higher or 0.5 fold down (lower) were defined as differential expression. Semi-quantitative RT-PCR was used to validate the microarray results. **RESULTS**: Among the 447 metastasis-associated genes, 4 genes were up-regulated and 14 genes were down-regulated in 3AO cells treated with aspirin for 16 h compared with untreated cells. While 24 genes were up-regulated and 10 genes were down-regulated in cells treated with aspirin for 48 h. Several up or down-regulated gene expression changes continued from 16 h to 48 h. **CONCLUSION**: Aspirin might exert its anti-metastasis effects on ovarian cancer by affecting metastasis-associated gene expression.

INTRODUCTION

Aspirin and other agents characterized as non-steroidal anti-inflammatory drugs (NSAIDs) are designed primarily to decrease pain and inflammation. As it can inhibit platelet aggregation and coagulation, aspirin is also used to prevent myocardial and cerebral infarction. It has been shown that long-term use of NSAIDS can reduce the incidence and mortality of colon cancer by 50 % and also reduce the numbers and size of colonic familial adenomatous polyposis (FAP)[1,2]. Aspirin has been confirmed to exert its anti-cancer effects by COX-independent mechanisms by resulting in an increase in mismatch repair (MMR) protein expression and subsequent apoptosis[3]. To investigate the mechanism by which aspirin exerts its anti-matastasis effect and to promote specifically its use in the treatment for ovarian cancer, the effect of aspirin on the metastasis-associated gene expression was studied by microarray in 3AO ovary cell lines.

MATERIALS AND METHODS

Microarray fabrication Total of 447 cDNA clones were obtained from Research Genetics [Invitrogen, Life technologies, USA]. *E coli* clones were cultured with Luria-Bertain culture medium supplemented with ampicillin (50 mg/L in final concentration)

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or chloromycetin (170 mg/L) in Innova™ 4330 refrigerated Incubator shaker(New Brunswick Scientific, USA) at the speed of 250 r/min overnight at 37 °C. Clone plasmids were extracted with Edge BioSystems (Gaithersburg, MD, Germany). Clone inserts were PCR-amplified from the plasmids with M13 vector specific universal primer (M13F: 5´-GGT GTA AAA CGA CGG CCA GTG-3´; M13R: 5´CAC ACA GGA AAC AGC TAT G-3´) in 96-well PCR microtiters. The PCR products were purified with protocols published[4], and resuspended in Arrayit spot solution. cDNA microarrays were fabricated on silylated slides (CEL Associates, Houston TX) with Cartisan PixSys 5500 robot (Cartesian Technologies, Irvine, CA) and UV- cross-linked at 3500 mJ using Cl-1000 Ultraviolet cross-linker. Microarrays were post-processed according to protocol online[5].

Cell culture and drug treatment 3AO cells were grown in the culture incubator at 37 °C with 5 % CO_2 in RPMI-1640 (Life Technologies, USA) supplemented with 10 % neonatal bovine serum. After the cells were cultured to 60 %-70 % confluent, 12 µL of aspirin dissolved in dimethyl sulpha-oxide (Me_2SO) was added to make the final concentration 1.2 mmol/L and further cultured for 16 h and 48 h, respectively. The same amount of Me_2SO was added to the control.

RNA extraction 3AO cells treated with aspirin or Me_2SO were lysed with Trizol (Life Technologies Inc, Rockville, MD, USA) according to the manufacture's protocol and total RNA were extracted and stored at -80 °C. The concentration of total RNA were measured with Biophotometer (Eppendof AG22331, Hamberg, Germany) and the 260/280 ratio of RNA was 1.8 to 2.0.

Probe preparation Probe preparation was done as described online protocols[6] and as shown by Yang *et al*[7] with some modifications. First strand cDNA was synthesized by priming 10 µg total RNA with 6 µg random hexamers (Life Technologies Inc, Rockville, MD, USA) by heating at 70 °C for 10 min, snap-cooling on ice for 30 s and placed at room temperature for additional 5-10 min. Reverse transcription was performed in the presence of 500 µmol/L each of dATP, dCTP and dGTP, 200 µmol/L aminoallyl-dUTP (Sigma Chemical Co, St Louis, MO, USA), 300 µmol/L dTTP, 1×first strand buffer, 10 mmol/L dithiolthreitol, and 400 U superscript II (Life technologies) in 30 µL reaction at 42 °C overnight. Reactions were quenched with EDTA 0.5 mol/L and RNA template was hydrolyzed by addition of 10 µL NaOH of 1 mol/L followed by heating at 70 °C for 10min. Reactions were neutralized with 10 µL 1mol/L HCl and cDNA was purified with Amicon Microcon YM100 (Millpore Corporation,Bedford, MA01730, USA) according to the manufacture's protocol. cDNA was lyophilized in speed vaccum concentrator 5301 (Eppendorf, Germany) and resuspended in 4.5 µL sodium carbonate buffer 0.1 mol/L (pH 9.0). Aliquot of Cy3 NHS ester dye (Amersham Pharmacia Biotech, England) was dissolved in 4.5 µL Me_2SO (1 mg dye from one tube was dissolved in 73 µL of Me_2SO and aliquot in 16 tubes and dry in speed vaccum and stored at 4 °C) and added to the resuspended cDNA and reactions were incubated at room temperature in the dark for 1 h. Coupling reactions were quenched by addition of 41 µL sodium acetate (pH 5.2) 0.1 mol/L, and unincorporated dye was removed using QIAquick PCR Purification Kit (QIAGEN, Germany) as manufacturer's instruction.

Hybridization and image processing Each slide was printed with duplicate microarrays. Slides were pre-hybridized in 1 % BSA, 5×SSC, 0.1 % SDS for 45 min, washed by dipping in de-ionized double distilled H_2O and 2-propanol for 2 times and air-dried and used in 1 h. Fluoroscent cDNA probes were lyophilized to dryness and resuspended in 10 µL hybridization buffer (formamide 5 µL, 20×SSC 2.5 µL, reagent grade double distilled water, (RGDD H_2O) 1.0 µL, 2 % SDS 0.5 µL and human cot-1 DNA 1 µL). Probes were denatured at 100 °C water bath for 2 min and cool at room temperature for 5 min. Room temperature probes of aspirin treated and untreated group were applied to the duplicate microarrays on the same pre-hybridized microarrays and covered with hybridized coverslip (Sigma) and placed in the Hybridization chamber (Corning). Hybridizations were carried out at 42 °C water bath for 20-22 h followed by washing in 2×SSC and 0.1 % SDS for 3 min, 1×SSC for 2 min and 0.2×SSC for 1 min and 0.05×SSC for 10 s, and dry by spin at horizontal plate centrifuge at 90×*g* for 4 min. Microarrays were scanned using a ScanArray 4000 (Packard Biosience, PE, USA) dual color confocal laser scanner. Data were saved as paired TIFF images.

Data analysis Spots were identified and local background substracted in the QuantArray 3.0 rendered by the manufacturer. In the first step, a grid consisting of square cells is drawn around each array element. Spot segmentation was then performed using a fixed segmentation method that uses the distribution of pixel intensity to separate probable signal from background and a binary threshold approach to identify spots, followed by a procedure to exclude disconnected features.

Raw intensity for each element was obtained by first excluding saturated pixels, then summing all remaining pixel intensities inside the spot contours. The area outside the spot contour but inside the cell was used to calculate local background. Background per pixel was estimated as a median of the pixels in this area and was multiplied by the spot area to give an estimated spot background value. In the final step, this integrated background value was substracted from the raw integrated spot intensity to produce the background-substracted integrated intensities used for further analysis. Furthermore, a quality control (QC) filter is used to remove questionable array features. Two criteria for spot rejection are spot shape that deviates greatly from a circle and low signal to noise ratio. Spots for which the ratio of area to circumference deviates by more than 20 % from the value for an ideal circle and spots containing fewer than 50 % of pixels above the median background values are flagged and eliminated from further consideration. This approach has proved extremely robust to misidentification of the spot boundaries and expression measures have shown it to be both reproducible and verifiable. The spot intensity that was above blank plus 2SD was used for the final analysis. Then the data were normalized to total with software supplied by the manufacturer. The 2 fold up-or down-regulated genes were shown in red or green color respectively.

Semi-quantitative RT-PCR RT-PCR was performed on Perkin-Elmer Applied Biosystems PCR heat cycle using TaKaRa two-step reaction with protocols supplied by the manufacturer. Primers were designed in the Primer 3 old Version[8], the primers were as following:
RAB2: forward: 5´-ACATCATAATCGGCGACACA-3´,
Reverse: 5´-GAGTCCATGTTCTCGTGCAA-3´
β-actin: forward: 5´-TGGARTCCTGTGGCATCCA-3´,
Reverse: 5´-CGCTCAGGAGGAGCAATGAT-3´. The PCR reaction was performed at 94 ℃ for 2 min, and 30 cycles at 94 ℃ for 30 s, 56 ℃ for 30 s and 72 ℃ for 30 s and final extension at 72 ℃ for 5 min. PCR products were electrophoresed in 1 % agarose gel. Images were captured with Alpha Image™ 2000. The band density was measured with the software rendered by the same system.

RESULTS

Plasmid extraction The plasmids were extracted by Edge Biosystems Plasmids Extaction kit (Gaitherberg, MD, Germany). The extracted plasmids were run on 1 % agarose gel. The results shown in Fig 1 were representative of the 447 clones. The PCR amplification of the 447 Clones for the inserts were showed in Fig 2. The single band successfully amplification rate were 93 %. There were 2.4 % of the reaction yielding multiple or weak bands, there were 3.3 % failed to amplify. It was similar as Genome research.

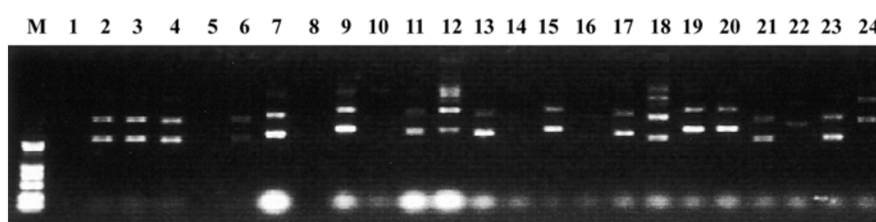

Fig 1. Electrophoresis of clone 73-95 plasmids in 1 % agarose. The missed wells were re-extracted and supplemented to the associate wells and used for PCR amplification for preparation of cDNA microarray. M: DL2000, lane1-24 represent plasmids of clone 73-95.

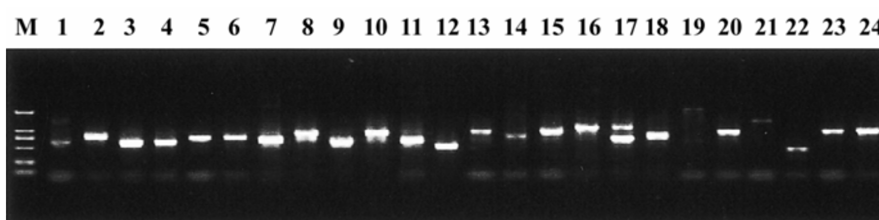

Fig 2. Electrophoresis of PCR products in 1.5 % argose used for preparation of microarray. M: DL2000. Lane 1-24 were the PCR amplification of clone 73-95. The missing lanes were re-amplified and replaced in the wells in 96-well PCR plate.

Some clones were sequenced by Biosia company in Shanghai (data not shown). Included in the microarray were also 7 housekeeping genes and 3 plant genes.

To identify metastasis associated genes affected by aspirin, 3AO cells were treated with aspirin 1.2 mmol/L for 16 h and 48 h, respectively. A representative image of gene expression of aspirin treated and untreated 3AO cells were shown in Fig 3. Each gene has duplicate. Control gene expression was shown in Fig 3A and gene expression of aspirin-treated cells was shown as Fig 3B. The overlay image of aspirin treated cells to untreated cells was shown in Fig 3C. The red spots represented up-regulated genes (≥2 fold) and green represented down regulated genes (≤0.5 fold.) and yellow spots represented genes expression difference were between 0.5 to 2.0. After normalization genes exhibiting a consistent 2-fold up- or 0.5 fold down-regulation across three replicates were considered significant. After treatment with aspirin for 16 h, there were 4 genes up-regulated and 14 genes down-regulated (Tab 1). The RAB2 gene was up-regulated by the aspirin and this up- regulated effect was still present at 48 h. In the down-regulated genes, there were anti-chymotrypsin and butyrophillins that were still down-regulated at 48 h. At 48 h there were more genes regulated by aspirin. Of the 447 genes analyzed by microarray, there were 24 genes up-regulated and 10 genes down-regulated (Tab 2). The up-regulated genes included signal transduction molecules (RAB2, fer, EGFR), nuclear remodeling components (HDAC 1, MTA1) and several ESTs.

Validation of the microarray results by semi-quantitative RT-PCR RAB2 gene expression was validated with TAKARA version 2.1 RT-PCR Kit. The expression level was measured by Alpha InnoTech™ 2000 spot density method. The aspirin treated 3AO cells expressed more RAB2 mRNA (Fig 4). The relative expression ratio to β-actin were 1.59, 4.07, 4.22 for the 3 replicate experiments respectively (lane 2-4). It was concordance with the microarray results.

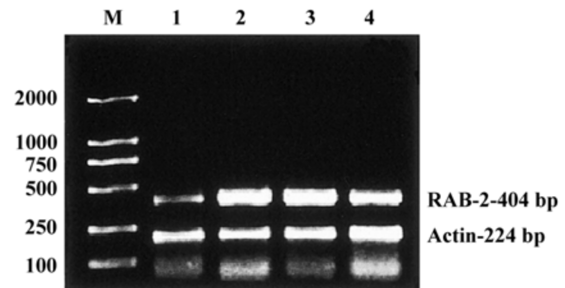

Fig 4. Semi-quantitative RT-PCR validating for up-regulated gene RAB2 (48 h). RT-PCR was used to detect the mRNA expression of RAB2 in treated or untreated 3AO cells. The RT-PCR products were run on 1 % agarose gel. M is DNA molecular marker DL2000. Lane 1 is mRNA expression of untreated 3AO cells. Lanes 2-4 are RAB2 mRNA expression levels in three replicates of aspirin treated 3AO cells.

DISCUSSION

cDNA microarray analysis is a high-throughput method to identify target genes of drug. For example, two genes named PSMB6 and ITGB1 have been identified as arsenic trioxide target genes in NB4 cells[8]. This method can also be used to identify targeted genes of Chinese herbal ingredients. MAPKKK and PI3K had been identified as triptolide target genes in Jurkat cells[9].

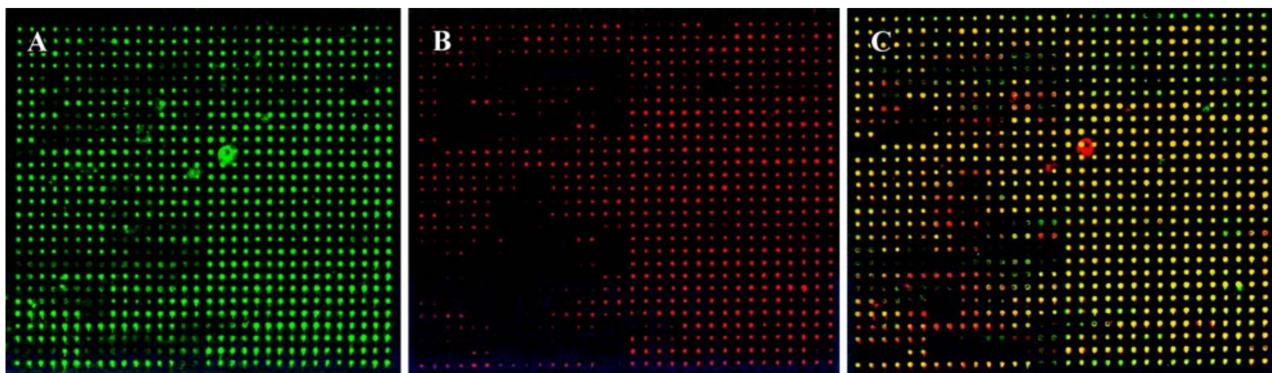

Fig 3. Representative Image of metastasis associated genes of microarrays: A) Confocal Scan array Image of untreated 3AO cells. B) Image of aspirin treated 3AO cells. C) Overlay Image of 3AO treated cells to untreated cells. All clones were spotted in duplicate. Column 15-16 in row 28 in each image was negative control. Yellow spots represent expression of the genes in aspirin treated were below two-fold up or down to control and green spots represent down-regulated and red spots represent up-regulated genes expressed in aspirin treated cells compared to control.

Tab 1. Genes up-regulated in 3AO cells treated by aspirin 1.2 mmol/L for 16 h. *n*=3 experiments. Mean±SD.

Accession number	Gene name	Ratio
BC059944	Hypothetical protein (244146)	2.32±0.17
BC008929	RAB2, member RAS oncogene family (34014)	2.34±0.40
T94037	Pyruvate kinase, muscle (119533)	2.19±0.23
BE679885	ESTs ,Weakly similar to Strabismus [D melanogaster] (113954)	2.73±0.14
K01500	Alpha-1-antichymotrypsin (3024341)	0.34±0.12
BI390905	KIAA0088 protein (2494032)	0.56±0.18
BC020214	Butyrophilin, subfamily 3, member A2(2494863)	0.50±0.16
BC008005	Nucleotide binding protein 2 (*E coli* MinD like) (2498589)	0.44±0.28
Not found	ESTs (2498958)	0.38±0.10
Not found	ESTs (2511666)	0.58±0.11
BC051907	Fragile X mental retardation, autosomal homolog 2 (2134952)	0.61±0.21
BC011714	Heterogeneous nuclear ribonucleoprotein D-like (1706718)	0.56±0.08
BC022512	Platelet/endothelial cell adhesion molecule (CD31 antigen) (PECAM1) (121164)	0.55±0.11
AF071400	Plasminogen activator inhibitor, type II (arginine-serpin) (PAI2) (323255)	0.58±0.17
NM006988	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1 (ADAMTS1) (2784234)	0.51±0.19
NM001953	Endothelial cell growth factor 1 (platelet-derived) (ECGF1) (3879593)	0.53±0.22
NM005246	Fer (fps/fes related) tyrosine kinase (phosphoprotein NCP94) (FER) (3886018)	0.52±0.12
NM080682	Vascular cell adhesion molecule 1 (VCAM1) (3893583)	0.55±0.15

The number in parenthesis represents IMAGE Clone Number.

To identify the metastasis-associated genes affected by aspirin, we analysed the expression of metastasis-associated genes in 3AO ovarian epithelial carcinoma cell lines with cDNA microarray which included oncogenes, tumor suppressor genes, adhesion molecules and signal transduction components and some expression sequence tags (ESTs). The gene expression profiles were detected at 16 h and 48 h after aspirin treatment. The results showed that at 16 h after treatment there were 4 genes up-regulated and 12 genes down-regulated. The up-regulation of RAB2 gene was still in high expression level at 48 h. It had been reported that a component from a Chinese herbs called homoharringtonine (HHT) had up-regulated effects on RAB14[10]. This indicated that small molecule drug may affect the tumor cell growth by affecting the expression of signal pathway components. In the 12 down-regulated genes, 3 genes were related to adhesion molecules, including PECAM1 (CD34), PAI2 and VCAM1. These genes were all related to the metastasis and invasion of tumor cells. This may explain why aspirin was used as anti-metastasis reagents. It also reduced a tyrosine kinase (fer) which was a signal transduction protein. It has been reported that this gene was a growth factor components. Its down-regulation on alpha-1-antichymotrypsin was also continued after 48 h treatment. After a 48 h treatment, 24 genes were up-regulated by aspirin on 3AO cells including 12 known genes and 12 ESTs. The 12 known genes included hemoglobin gamma G, UDP-Gal: β-GlcNAC-β1,4-galactoayltransferase, polypeptide1, β_2-microglubulin, RAB2, member ras family, ribosomal protein 19, histone deacetylase 1 (HDAC1), pulmonary surfactant associated protein d precursor, heat shock 70kD protein1A, EGFR (v-erb-b), MTA1, and NM-23-H6.

It is known that the product of MTA1 and histone deacytylase 1 are all the components of nucleosome remodeling complex (NuRD) and it is over-expressed in a variety of human cell lines (breast, ovarian, lung, gastric and colorectal cancer but not melanoma or sarcoma) and cancerous tissues (breast, esophageal, colorectal, gastric and pancreatic cancer). It encodes a novel protein that contains a proline rich region (SH3-binding motif), a putative zinc finger motif, a leucine zipper motif and 5 copies of the SPXX motif found in gene regulatory proteins. Experimental inhibition of MTA1 protein expression using anti-sense phosphorothioate oligonucleotides resulted in inhibition of growth

Tab 2. Genes up-regulated and down-regulated by aspirin 1.2 mmol/L in 3AO cell for 48 h. *n*=3 experiments. Mean±SD.

Accession number	Gene name	Ratio
NM000184	Hemoglobin, gamma G (186624)	2.43±0.36
BE683774	ESTs (183708)	2.08±0.25
Not found	ESTs (30374)	2.34±0.33
BC045773	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1(2134956)	2.34±0.33
BQ271358	ESTs, Moderately similar to COLLAGEN ALPHA 1(XII) CHAIN [R norvegicus] (2144643)	1.88±0.29
AI131085	ESTs (1706278)	2.47±0.33
U51903	ESTs (1262925)	2.29±0.52
BM023409	ESTs, Moderately similar to pseudouridine synthase 1 [H sapiens] (1401185)	2.50±0.27
AA640389	ESTs (1203795)	2.43±0.40
Not found	ESTs (1171064)	1.88±0.21
AA642168	ESTs (1154853)	2.51±0.21
BC032589	Beta-2-microglobulin (544335)	2.35±0.43
AA592615	ESTs (1051255)	2.23±0.48
AA139649	ESTs (585961)	2.23±0.34
CB432767	ESTs (609016)	2.04±0.30
BC008929	RAB2 member RAS oncogene family (728947)	2.04±0.30
BC007615	Ribosomal protein S19 (728984)	2.49±0.23
NM001022	Ribosomal protein S19 (762885)	2.03±0.26
NM004964	Histone deacetylase 1 (HDAC1) (3875757)	2.17±0.40
BE465060	Pulmonary surfactant-associated protein d precursor (human) (2746772)	2.34±0.39
NM005345	Heat shock 70kD protein 1A (HSPA1A) (2782837)	2.17±0.32
NM005228	Epidermal growth factor receptor (avian erythroblastic leukemia viral (v-erb-b) oncogene homolog) (EGFR) (2783652)	2.30±0.41
NM004689	Metastasis associated 1 (MTA1) (4054392)	2.38±0.36
BC012828	Nucleoside diphosphate kinase type 6 (inhibitor of p53-induced apoptosis-alpha) (NM23-H6) (2966629)	2.35±0.32
BC002832	Butyrophilin, subfamily 3, member A2 (1070574)	0.64±0.15
Not found	ESTs (2511666)	0.44±0.12
BC008005	Nucleotide binding protein 2 (E coli MinD like) (2498589)	0.54±0.14
BC020214	Butyrophilin, subfamily 3, member A2 (2494720)	0.63±22
BC020214	Butyrophilin, subfamily 3, member A2 (2494863)	0.55±0.21
K01500	Alpha-1-antichymotrypsin (3024341)	0.45±0.06
BC028178	Collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant) (116863)	0.51±0.07
BC053521	Spectrin, alpha, non-erythrocytic 1 (alpha-fodrin) (124959)	0.55±0.19

The number in parenthesis represents the IMAGE Clone number.

and invasion of human MDA-MB-231 breast cancer cells with relatively high MTA1 expression. MTA1 protein may serve multiple functions in cellular signaling, chromosome remodeling and transcription processes that are important in the progression, invasion and growth of metastatic epithelial cells[11]. Aspirin can enhance the expression of these two genes at the same way. It has been reported that another NSAIDs sulindac also had up-regulating effects on the MTA1 expression[12], whereas MTA1 genes in 3AO/cDDP were down-regulated by arsenic trioxide[13]. MTA1 had been reported up-regulated in ovarian carcinoma and correlated with tumor metastasis and hepatocellular carcinoma and not correlated with invasion but correlated with the overall survival[14,15]. The metastasis-associated protein MTA1 has been shown to express differentially to high levels in metastatic cells[16]. The dynamic balance between histone acetylation and deacetylation plays a significant role in the regulation of gene transcription. The inhibition of HDAC up-regulated RhoB expression and provided an important insight into the mechanisms of HDAC-mediated transcriptional control and the poten-

tial therapeutic benefit by HDAC inhibition[17]. HDAC1 acted as an antagonist of the tumor suppressor p53 in the regulation of the cyclin-dependent kinase inhibitor p21 and provided a basis for understanding the function of histone deacetylase inhibitors as anti-tumor drugs[18].

Aspirin also inhibited the PAI2 expression. It has been shown that the PAI-2 expression was correlated with low overall survival[19]. PAI2 can combine with PA receptor and has been used as a carrier of 213Bi radio- active therapy agents[20]. Fer protein expression was enhanced with prostate cancer cell proliferation and enhanced in prostate cancer. Aspirin can inhibit its expression[21]. Aspirin also up-regulated heat shock protein 70 kD protein 1A. Its significance on these genes needs further investigation.

In conclusion, our results showed that aspirin may affect many genes expression and its anti-cancer effects might be related to genes involved in tumor metastasis and invasion.

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