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Case Report

A Therapeutic Targeting Identification from Microarray Data and Quantitative Network Analysis

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Abstract. Personalized therapy is "the right drug for the right patient at the right time". Here we reported a case of personalized therapy using gene expression signature (GES) related drug discovery to treat a patient with drug-resistant metastases from breast-tumor. *Methods***:** After mRNA obtained from metastatic liver tissue was performed by microarray, GES of genomic profiles were uncovered by bioinformatics tool and targeting drugs related with GES were mined by drug-bank. Several targeting-drugs approved by FDA were selected to treat the patient. *Results***:** 1198 genes were uncovered for the higher expression by two-fold to compare normal liver specimens in which 10 of mined genes were identified as set-1 GES for metastasis and 16 of genes were uncovered as set-2 directly for primary breast tumor. Drug-bank platform were used to discover drugs for target set-1/2 genes. Eventually, medropxyoprogesterone (MPA) targeting set-I gene and doxorubicin targeting set-2 gene were selected for the patient because the two drugs have already been approved by FDA. After doxorubicin and MPA were administered, patient's metastatic-tumor showed complete response. *Conclusions***:** We not only analyze genomic expression profiles but also discover sensitive compounds for drug-resistant tumor. We successfully select drugs approved by FDA to treat the patient.

Keywords: Genomic expression profile; GEO profile; gene expression signature (GES); quantitative pathway; drug discovery; Doxorubicin; Medroxyprogesterone; DDX21 (Nucleolar RNA helicase 21); Calcyclin (S100 calcium binding protein A6)

1. Introduction

Clinical genome analysis has the ability to provide some information required for identification of genotype signature (GWAS), gene expression signature (GES) and drug discovery [1–4]. Enormous amounts of genomic analysis have been used for personalized therapy (the right drug for the right patient at the right time) of different tumor diseases, genetic diseases and unknown rare diseases. These kinds of genomic analysis and diagnosis always use DNA, RNA and proteins from a pair of tissues or cells including a pair of surgical specimens such as tumor tissue *vs* normal tissue by

in vivo sampling [5], a pair of pathological specimens such as a pair of tumor cells *vs* normal cells *in situ* sampling obtained from laser capture microscopy (LCM) [6, 7] or fresh cells from clinical specimens by *ex vivo* culture [8]. Such clinical genomics database, when combined with quantitative genomic analysis, allow physicians and scientists to identify genotype signature, gene expression signature and discover drugs for patients with drug resistance in tumor disease or unknown treatment in genetic, neurological and rare diseases [9, 10]. One of the major challenges we often face is how to obtain a pair of specimens. Here we introduce a mining process from microarray data obtained from lonely liver metastatic tissue with downstream quantitative genomic analysis for identifying GES and discovering drugs related to GES. Following a three-step process, that is, mining genomic expression profile, identifying gene expression signature and discovering specific drugs, a set of drugs specifically targeting metastasis liver (set-1) and specifically targeting primary tumor (set-2) are used for the patient.

2. Methods

2.1. Patient and specimen. The patient was given diagnoses according to clinical criteria. Informed consent was obtained by the patient for the genomic analysis and drug targeting. Tumor tissue (snap-frozen in liquid N2) were obtained at the time of surgery and were stored at 80˚C. Metastasis into liver from breast ductal tumor was diagnosed and classified according to cell type by conventional pathology.

2.2. Microarray experiment. Microarray was performed on RNA derived from the patient liver metastasis specimen. RNA extract and microarray process were prepared according to the manufacturer instructions (Affymetrix Expression Analysis Technical Manual; Affymetrix, Santa Clara, CA) [11, 12]. Briefly, RNA specimens triple from liver metastasis were extracted by Trizol reagent (Invitrogen, Carlsbad, CA) and cleaned by RNaeasy column (Qiagen, Valencia, CA). After sequential washing, total RNA was eluted in RNase-free water. Isolated total RNA was quantified and its integrity was confirmed on a 2100-Bioanalyzer. Each 1ug of triple RNAs was used to prepare biotinylated antisense RNA (cRNA) using Ambion's MessengeAmpII-Biotin Enhanced kit (Ambion, Austin, TX) and 15ug of fragmented biotinylated cRNA was hybridized to each Genechip Human Genome U133-2A for the triple experiment.

2.3. Genomic data mining. According to current clinical genomic methods, at least three ways can be used as mining for heterogeneous cells, which are hierarchical cluster, principle component analysis (PCA) and self-organizing map (SOM) [13, 14]. In these analyses, after normalization of microarray expression data by Model-based Background Correction (MBCB) combined by MAS5 (which have been established by our colleagues) [15], hierarchical clustering and significance of microarray (SAM) would be used to uncover genomic profiles. All of hierarchical clustering and SAM are performed by BRB platform and NIA platform from NIH as our previous reports [16, 17]. Briefly, in order to mine specific gene profile only from metastasis, we first compare triple normalized profiles of the patient specimens to normalized profiles of three normal liver tissues (from GEO) by SAM and Hierarchical clustering performance with cut off two-fold increase. After genomic profiles were uncovered, the profiles of patient specimens should still have mixed genes from vessel cell or lymphocytes or macrophages because of the raw microarray data from tissue level. In order to exclude these kinds of genes, we also conduct merging analysis in database of T-cells and macrophage which have been stored in our database as our previous report [18]. Following excluding the mixtures, eventually, a set-1 profile were classified as liver-metastasis profile by merging the profile to triple GEO data which is as similar as liver metastasis spreading from breast ductal tumor and a set-2 profile was defined as profile of primary ductal breast cancer by comparing genomic profile of patient to genomic data from primary ductal breast cancer in GEO.

2.4. Identification of gene expression signature: After we harvest genomic profile as set-1 and set-2, in which some or most of them cannot be directly used as drug targeting. We continue identifying GES in which some genes have higher linking with most or all of tumor cell function. Our quantitative analysis of gene network topology was focused on "betweenness" and "connectivity" which was identified by Python scripts developed by ourselves based on the two indices, that is, higher betweenness and low connectivity [19, 20].

2.5. Drug discovery. Following the quantitative analysis of pathways, GES of set-1 genes for specific hepatic metastasis and GES of set-2 for specific primary breast tumor are input into GeneGo software and drug-bank [21, 22] to discover drugs. Finally, set-1 drugs were discovered for hepatic metastasis and set-2 drugs used for targeting primary breast tumor.

2.6. Software availability. Genomic data mining from liver tissues were conducted by BRB platform as our previous report [15]. GES determination is processed by our own Python scripts for therapeutic identification. GeneGo software and drug-bank were used for drug discovery. Some public data such as GEO are also used in the project.

3. Results

3.1. Patient. The patient is 55 years old female, 65 kg. In 2007, right breast ductal tumor (with immunochemical

staining diagnosed as triple-negative breast tumors, or negative for estrogen receptor, ER, progesterone receptor, PR, and HER2) was removed with six courses of chemotherapy (cisplatin and docetaxel) and one course of radiation. Six courses chemotherapy and one course radiation were performed against spinal metastasis in 2009. One course of interventional therapy was processed against liver metastasis in 2011. In 2012, new metastasis in right liver and multiple lymph nodes have recurrence so that the patient was selected as candidate for personalized therapy.

3.2. Microarray results. In order to decrease bias from chip types, we selected Affymetrix human genome U133-2A as chip assay because several normal liver database performed by U133-2A microarray (GSM362951, GSM362953, GSM362955) have been stored in GEO database. After microarray performance and triple repeat data come out, MBCB combined MAS5 was used for normalization by both patient's database from metastatic tissue and GEO database from normal liver. These performances using similar array chip can greatly decrease bias those from different chips. Significant analysis of microarray (SAM) was used for mining genes as a whole mining plan (Figure 1A). As supplemental Table 1, total 1198 genes are two-fold higher in the patient than those from normal liver gene expression in GEO database. Hierarchical clustering analysis confirmed gene expression pattern (Figure 2).

3.3. The results of genomic mining. After 1198 genes were uncovered for higher expression in patient metastatic tumor, the raw genes may still have chances for mixed some genes with higher expression levels from lymphocytes (TIL) and macrophage in the tumor tissue. In order to study these genes, we employed specific database of T-cells and macrophage which all have been saved in our database as our previous report [18]. Fortunately, no genes regarding special-killing tumor cell such as TNF-alpha or FAS-L from T-cell or macrophage are mixed in the genomic expression profiles. In order to further classify the genome, we compared the 1198 gene profiles to GEO database with liver metastatic tumor spread from similar type of primary breast tumor (GSM352136, GSM3521139, and GSM352146). 253 candidate genes (set-1) were defined as set-1 genes shown in supplemental Table 2. Because of the metastatic mass from breast ductal tumor, in order to uncover specific genes from primary ductal breast cancer, we also compared the patient's genomic profile to database of both normal ductal tissues and breast ductal cancer from GEO (three normal ductal tissues for GSM134584, GSM134588, GSM134687, and three primary ductal cancer tissues for GSM134698, GSM134701 and GSM134704). 16 genes (set-2) as Table 1 have been uncovered as higher expression in breast ductal tumor to compare normal ductal tissues.

3.4. The results of identification of genomic signature. After harvesting genomic profiles, most of them cannot be directly used as drug targeting because we need identify effective therapeutic targets. Such analysis requires exploring network properties, in particular the importance of individual network nodes. There are many measures that use the importance of nodes in a network such as betweeness centrality (BC) and degree centrality (DC, connectivity). Although both high values may serve as effective drug targets, DC is also likely to be toxic due to their system-wide influence, thus we search therapeutic targeting with higher betweenness (>1.0%) for effective drugs and lower connectivity (<30) for lower toxicity due to lower system-wide influence. As in Table 2, 10 of 253 proteins from set-1 profile was identified as "gene expression signature" by computational analysis with our Python script based on the quantitative pathway topology for targeting patient metastatic tumor.

3.5. Drug discovery. After identifying set-1 gene expression signature for specific hepatic metastasis and set-2 for specific primary breast tumor, all targeted genes are input into Gene-bank database, several drugs were discovered to specifically target hepatic metastasis and primary breast tumor as Table 3. As shown in Table 3, doxorubicin is specific to targeting DDX21 and GSK923295 is to repress CENP-E of set-1 genes. Medroprogesterone is to inhibit calcyclin and pentamidine is to specifically attack matriptase of set-2 genes, respectively. Interestingly, secobarbital and pentobarbital which are not used for tumor diseases are also discovered to inhibit RacGAP from set-2 proteins. In order to visualize the targeting identification of GES related with drug discovery, a mapping identification of expression signature from primary breast tumor is illuminated as in Figure 3A and Supplemental-Figure 3A and 3B. A mapping identification of expression signature from metastatic liver is displayed as shown in Figure 3B and Supplemental-Figure 3C and Figure 3D.

3.6. Clinical application and results. According to mining GES and uncovering drugs, finally selected drugs to treat the patient tumor disease are relied on drugs approved by FDA and clinical comprehensive consideration. Because doxorubicin has been approved by FDA to treat advanced breast tumor and MPA have also been approved by FDA to treat some woman diseases as in Supplemental Table 3. Some clinical data show large dosage administration of MPA is much better than small dosage administration of MPA [23]. Furthermore, combination of doxorubicin with docetaxel and cyclophosphamide shows better response than doxorubicin treatment alone [24–27]. Five courses of doxorubicin with docetaxel and cyclophosphamide and daily MPA management are performed for the patient (see Table 4). After sequencing and combination treatment, as Figure 4A and 4B, metastasis in right liver and multiple lymph nodes have partial or complete responses after the 5 months'

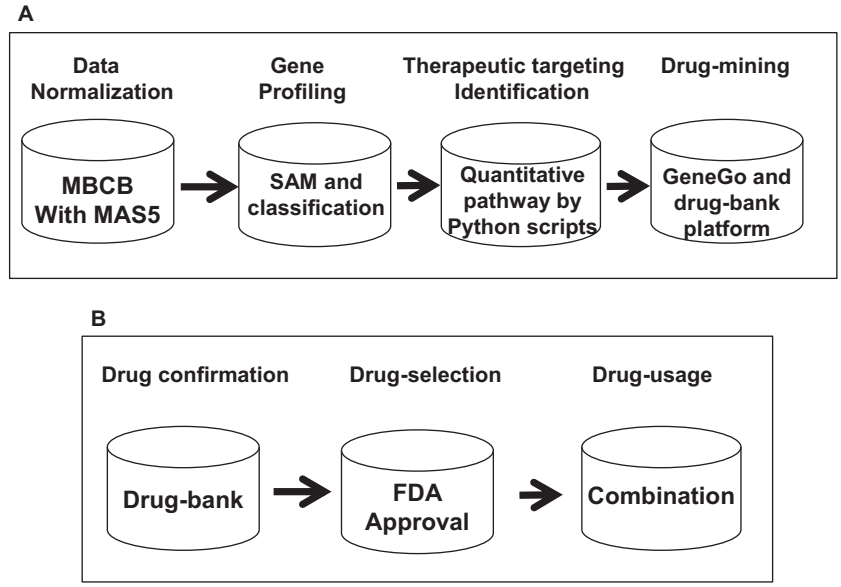

Figure 1: Strategies of therapeutic targeting identification and drug discovery for personalized therapy. Shown is a schematic diagram outlining our genomic bioinformatics approach for the data generated from microarray analysis as Figure1A and drug discovery and application as Figure 1B.

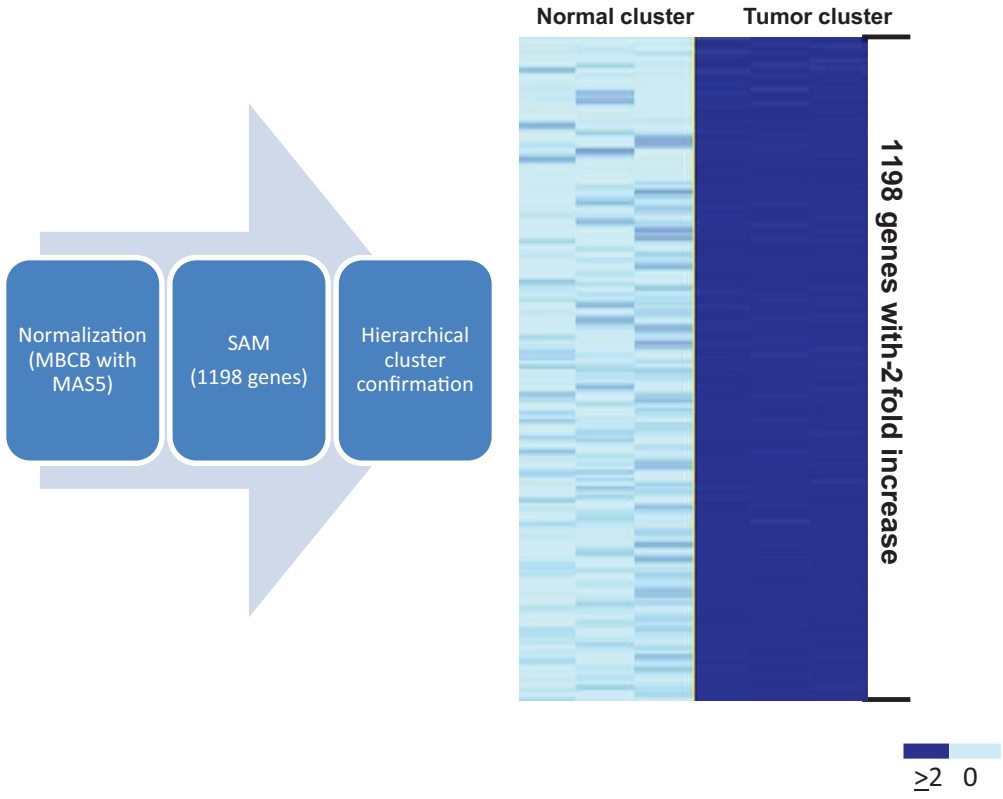

Figure 2: Strategies of gene profile mining. Expression values were normalized to the zero values at normal control cluster group to compare tumor cluster group with 1198 genes. The resultant log2 ratios were averaged and displayed in the heatmap. The deep color bar indicates log2 ratio of change higher than 2-fold increase. Light color is indicating the zero values as normal control in gene expression.

Table 1: Gene expression level from set-2 profile between breast tumor and normal breast duct.

Gene Symbol	Gene Expression fold-change (metastatic liver/normal liver)	Gene expression (log) from GEO of breast tumor			Gene expression (log) from GEO of normal breast duct		
		GSM134698	GSM134701	GSM134704	GSM134584	GSM134588	GSM134687
AGTPBP1	**3.43**	7.57	7.57	7.62	5.81	5.59	5.99
cct6a	**2.89**	9.09	8.91	8.95	6.98	7.4	7.95
CENPF	**12.48**	8.1	8	8.17	3.89	3.72	5.8
copb2	**2.36**	9.35	9.33	9.35	8.3	7.98	8.02
CUX1	**3.31**	7.59	7.42	7.71	5.7	5.44	6.29
DDX21	**3.45**	8.84	8.94	8.77	7.12	7.52	6.56
fam60a	**10.1**	9.15	9.08	9.06	5.32	6.01	5.91
ISG15	**12.25**	10.7	10.85	10.74	7.29	7.38	6.79
MXRA5	**6.41**	9.21	9.28	8.96	6.16	7.21	5.99
NCBP1	**4.28**	6.96	6.78	6.53	5.52	5.07	5.23
OSBPL10	**3.38**	6.2	6.4	6.41	3.8	5.01	4.94
PLP2	**6.39**	8.41	8.42	8.45	5.93	5.49	5.87
RBMS1	**3.59**	9.49	9.65	9.64	7.35	8.35	7.53
Sulf1	**47.15**	9.37	9.39	9.36	3.32	3.82	4.28
SYNCRIP	**6.25**	8.79	8.73	8.86	5.99	5.74	6.71
TAF1D	**6.73**	8.33	8.59	8.12	5.49	6.93	6.47

Table 2: Results of genomic expression signature from set-1 profile.

Gene Symbol	Patients Gene Expression Fold-change (metastatic liver/normal liver)	Connectivity	Betweenness
HSPA1A	3.41	6	0.015
HIST1H2BG	7.45	3	0.050
EIF3H	3.86	6	0.012
SET	3.50	21	0.042
ST14	4.72	5	0.158
S100A6 (Calcyclin)	16.41	6	0.021
RACGAP1	5.95	17	0.052
ORC3L	4.08	12	0.095
AGTPBP1	3.46	6	0.015
CBX5	3.64	28	0.126

personalized therapy with observation in the following 3 months. The patient also can be tolerant for side-effects (supplemental Table 4).

4. Discussion

Traditional clinical diagnosis and management focuses on the individual patient's symptoms, medical history, and data from laboratory and imaging evaluation to diagnose and treat illnesses. Recent new developments in human disease have provided us with a more detailed understanding of the disease in individual subject, such as, single nucleotide polymorphisms (SNP) and genome-wide association studies (GWAS) [28, 29]. Based on the new extension, targeting identification and drug discovery is an emerging clinical application area for drug-resistance tumor disease and unknown-treatment genetic disease and some rare disease, which is called as personalized therapy [30]. However, proteomics and transcriptome from genotype change (such as SNPs) may eventually have a great impact on the new medicine [31, 32]. Because the DNA genome is the information archive, their proteins and RNA do the work of the cell so that the functional aspects of the cell are controlled by and through proteins and RNAs, not gene's DNA level [33–35]. Moreover, most of the FDA approved targeted therapeutics are directed at proteins or RNAs, not DNA archives so that pharmaceutical interventions aim to modulate the aberrant protein activity, not genetic defect. In addition, because analyses of proteins have largely found

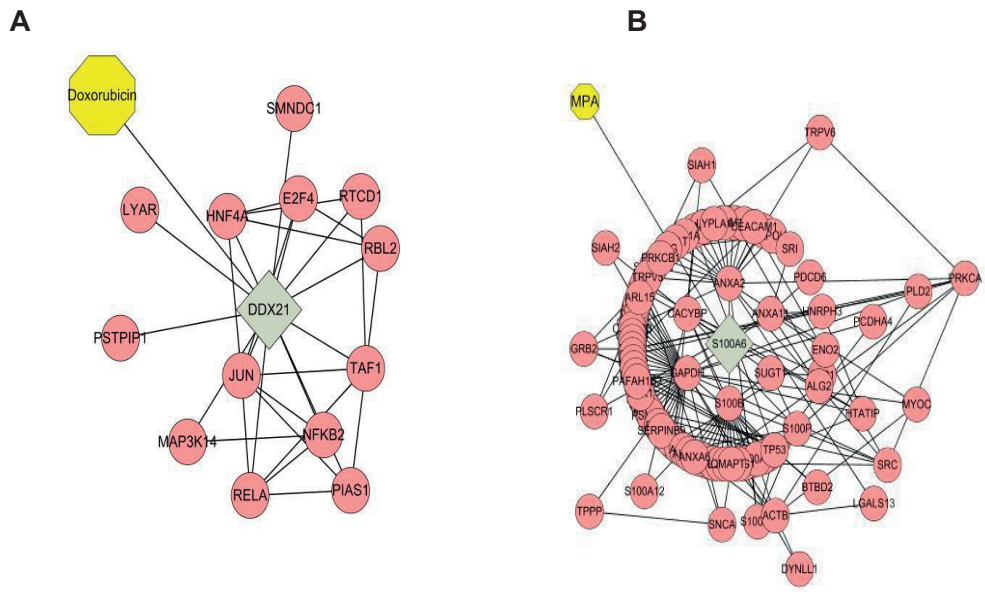

Figure 3: Analysis of pathway and network related with genomic expression signature and drug targeting. Figure 3A indicate S100A6 network from primary breast ductal tumor related with MPA and Figure3B show metastases pathway from DDX21 related Doxorubicin targeting.

Table 3: Result of drug discovery by GeneGo and Genebank platform.

Drug targeting	Drug names	Drug targeting proteins	Drug targeting function
Primary breast ductal tumor	GSK923295	CENP-E	Inhibition
Primary breast ductal tumor	Doxorubicin	DDX21	Inhibition
Liver metastasis	Medroxyprogesterone (MPA)	Calcyclin (S100A6)	Inhibition
Liver metastasis	Secobarbital	RacGAP1	Inhibition
Liver metastasis	Pentobarbital	RacGAP1	Inhibition
Liver metastasis	Pentamidine	ST14 (Matriptase)	Inhibition

little concordance between the SNPs archives and proteomics expression, clinical scientists now make an indirect analysis of the transcriptome to search a concordance between gene expression and DNA archives due to stable and feasible data and profiles from transcriptome levels such as RNA-seq and mRNA microarray [36, 37].

Triple-negative breast tumors (negative in estrogen receptor, progesterone receptor, and HER2) which do not respond to endocrine agents or some molecular therapy can only be treated with very few of drugs [38–40]. Fortunately, some of the newest treatment with triple-negative breast cancers has incorporated genomics analysis. Some scientists have presented an evaluation of triple-negative breast cancer according to genomic data [41]. It is hoped that advances in targeted treatment and optimization of chemotherapy based on genomic analysis will provide more effective treatment and improved outcomes for this aggressive subclass of breast cancer. The patient performed by traditional chemotherapy, radiation and interventional therapy is often recurrence, thus it is good candidate for us to perform personalized therapy relying on identifying gene expression signature and uncovering sensitive drug discovery.

For this case, tumor specimens obtained from surgery do not have enough condition to harvest normal control as negative control, which needs normal tissue 2 cm away from tumor mass so that we cannot use the tissue as both tumor specimens and normal control. The only way is to select GEO data from human normal liver as negative control (GSM362951, GSM362953, and GSM362955). Due to the three negative controls performed by Affymetrix human genome U133-2A, we also select U133-2A as chip performance for the patient specimens to decrease variability between different chips.

After microarray data was normalized, significant analysis of microarray (SAM) and Hierarchical cluster were used for mining gene expression profile. 1198 genes were higher expression by two-fold increase as current clinical genomic protocol to compare to normal liver specimens (although we have two-fold decrease profile, it is not important to relate GES so that they were omitted here). In order to

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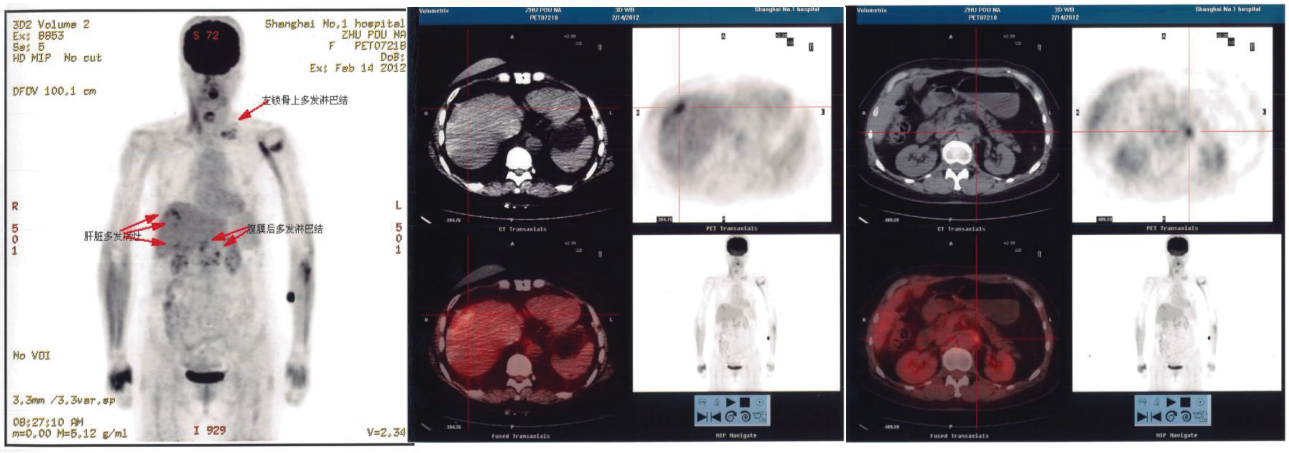

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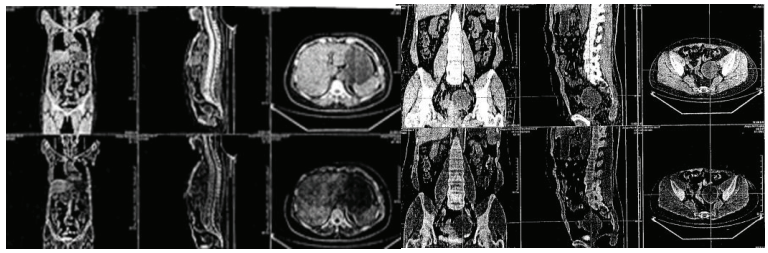
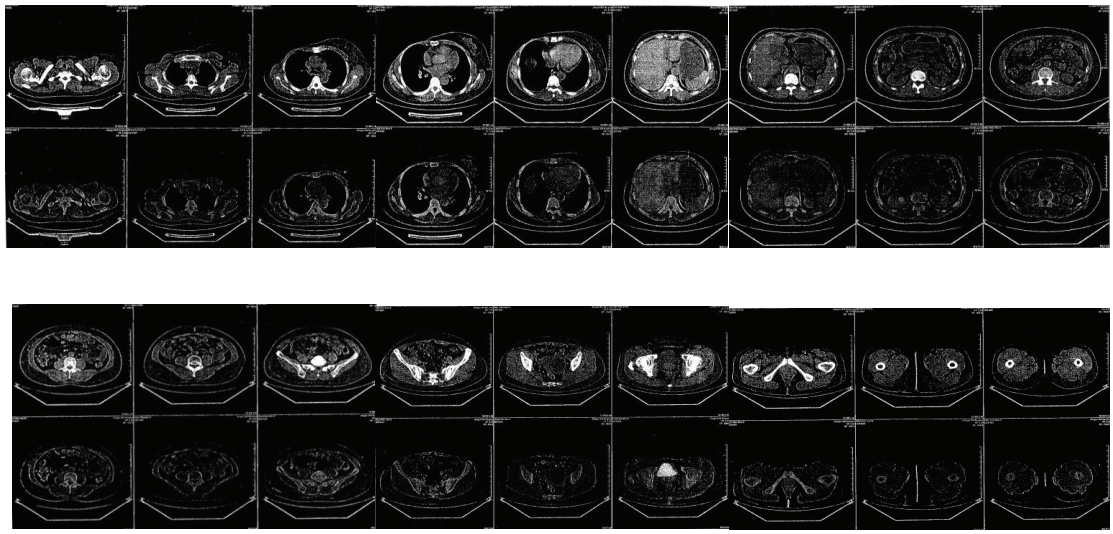


Non increase uptake of FDG



Mass disappearing and liquefaction

Figure 4: Results of positron emission tomography-computed tomography (PET/CT). Figure4A shows multiple metastases before personalized therapy. 4A1 indicating multiple metastasis in livers, multiple lymph nodes in subclavian and retroperitoneal position; 4AII show hypermetabolic areas by red masses with metastases in liver; 4AIII indicates hypermetabolic areas by red masses in retroperitoneal position. Figure 4B indicates good response after 5 months' personalized therapy.

Table 4: Drug treatment plan and application.

Drugs	FDA approval	Dosage	Application methods
MPA	FDA approved	4 x 250mg/daily/ 6 month	Oral
Doxorubicin	FDA approved	60 mg/m^2/21day/5 cycle	intravenous
Docetaxel	FDA approved	75 mg/m^2/21day/5 cycle	intravenous
Cyclophosphamide	FDA approved	500 mg/m^2/21 day/2 cycle	Intravenous

confirm the profiles, 1198 genes were compared to GEO gene profiles (triple database similar to liver metastasis of breast ductal tumor), finally 253 genes were mined for metastatic profile in the patient, called as set-1 genes. In order to search targeting profiles for breast ductal tumor, the 1198 genes were secondarily compared to GEO database of breast ductal tumor, a set with 16 genes was uncovered, called as set-2 genes.

After we defined genomic profile into set-1 and set-2, most genes cannot be directly used as drug targeting. The reason is that, although a gene is highly expressed in tumor cells, it might not be the key genes linking all functions in the tumor cell. That is, even if the genes are knockout in animal or knockdown in the cells, the cell maybe still survive because the cell function in the cell is not fully destroyed. On the other hand, if a genes linking all cell functions is discovered, the knockout of the protein in animal model or knockdown of it in cell model, will kill the cell. Based on the basic mechanism, we need work on identifying genomic expression signature by quantitative network analysis or topology. Now there are several quantitative network can be used for topology. Here "betweenness centrality" and "degree centrality", a routine topology to mine GES, are used for the analysis [42]. "betweenness centrality (BC)" is shortest pathway passing the gene and "connectivity or degree centrality (DC)" is how many genes linking the gene [43–45]. According to topology analysis, both all have effective influence cell function. Because DC is also likely to be toxic due to their system-wide influence, thus we define GES using higher BC and low DC [46]. Eventually, 10 genes of 253 genes selected from set-1 (with higher betweenness and lower connectivity from metastatic tissues) were identified for targeting metastasis tumor. 16 genes specifically targeting primary breast ductal tumor were selected for set-2.

Accordingly, two set of drugs were discovered to treat hepatic metastasis and breast ductal tumor from GeneGo software and GeneBank. Medropxyoprogesterone (MPA), pentamidine, pentobarbital and secobarbital are mined for targeting set-I genes and doxorubicin and GSK923295 are uncovered for targeting set-2 genes. Because MPA and doxorubicin have been approved by FDA for clinical application for different tumors (as Supplemental 3), MPA and doxorubicin are selected to treat the patient. Moreover, according to clinical comprehensive consideration, combination of doxorubicin is much better than lonely doxorubicin treatment so that doxorubicin is combined with docetaxel and cyclophosphamide to treat the patient. After five courses personalized therapy by combination of doxorubicin (5 courses), docetaxel (5 courses) *a*nd cyclophosphamide (2 courses) and MPA daily management, we successfully achieve a good response for the recurrence of metastatic liver and multiple metastatic lymph nodes for the patient.

5. Conclusion

After mRNA obtained from metastatic liver tissue was performed by microarray and GES were uncovered by quantitative network and targeting drugs related with GES was mined by drug-bank, several mined drugs which have been approved by FDA were selected to administer the patient. Finally, we discover selected drugs sensitive for the recurrence of metastatic liver and multiple metastatic lymph nodes.

Author's contributions

BL conceived, designed and guided the work and edited the manuscript. BL, HHL, DY supported clinical work. XNZ, JSH, HSX and QHZ performed experiments including RNA specimen preparation and microarray process. JZ and BL performed the bioinformatics analysis and drafted the manuscript.

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The inclusion of trade names or commercial products in this article was solely for the purpose of providing specific information and does not imply recommendation for their products.

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