Systems Metabolomic Study for Functional Characterization of Novel Oncogene in Breast Cancer

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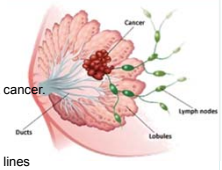
Abstract

Breast cancer is the most common cancer in women worldwide and also the most leading cause of death. In addition to the main risk factors (e.g. gender and age), other risk factors at the molecular levels have been known such as genetic mutation, abnormal hormone level, and obesity. Genotype has been recognized as an important key to link a hereditary and breast cancer syndrome (e.g. BRCA1,2 and ERBB2). However, it can partially explain disease occurrence and progress. In this study, we examined novel oncogene function and the metabolic consequences by applying mass spectrometry-based functional metabolomic approach. Accordingly, we acquired unique metabolite profiles from 4 different breast cancer cell lines and 2 different genotypes, and captured genotype-specific metabolite dynamics across each cell lines. Moreover, we explored the integrative analysis with metabolites and mRNA expression levels in order to clarify the role of novel oncogene in breast cancer.

Materials

Gene X

The gain of gene X (overexpression) demonstrates potential linkage to breast cancer.



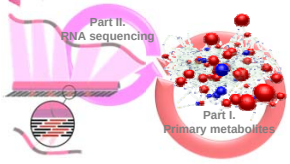
Cell Lines

Genetic effect of Gene X

1. Overexpression in normal breast cell lines
2. Knockdown using shRNA in breast cancer cell lines

Genotype	Cell line	Cell line information
Gene X overexpression	HMLE	Human mammary epithelial cells
	MCF10A	Non-tumorigenic human mammary epithelial cells
Gene X Knock-down	SUM149PT	Human breast tumor cell line
	SUM159PT	Human breast tumor cell line

Experiment design



Integrative Analysis

Metabolites analysis
1. Central carbon pathway
2. Noble compounds

+

NGS
(Next-generation Sequencing)
RNA Sequencing

Methods

Sample extraction and derivatization

1. Quenching : 1㎖ 70% Methanol in water(-70℃) + 1㎖ cell
2. Centrifuge and divided into cells and MeOH at 4 ℃
3. Lyophilized cells and dryness MeOH in a vacuum concentrator
4. Extract and disrupted cell and MeOH
   a. Using the ball mill MM301 (Retsch GMbH&Co., Germany)
   b. 5mm i.d. steel ball
   c. 750㎕ solvent (methanol : isopropanol : water, 3:3:2, v/v/v)
   d. Sonication 5min
   e. Centrifugation and aliquot → concentration and dryness
5. Derivatization
   a. Methoxyamination : 5㎕ of a solution of 40 mg/mL of methoxyamine -hydrochloride in pyridine → shaken at 30°C for 90 min
   b. Trimethylsilylation : 45㎕ of MSTFA and a mixture of internal -retention index (RI) markers, fatty acid methyl esters (FAME) → shaken at 37°C for 60 min

Mass-spectrometry analysis and Data processing

Agilent 7890B GC (Agilent Technologies, Wilmington, DE)
Pegasus HT TOF MS (LECO, St. Joseph, MI)

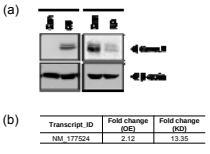
1. Injection : 0.5㎕, splitless mode
2. Oven temperatures : 50 °C for 1 min, followed by ramping to 330 °C at 20 °C/min, and a final holding for 5 min.
3. LECO Chroma TOF software (ver. 3.34; St. Joseph, MI) : GC/TOF MS data were pre-processed to detect peaks and deconvolute the mass spectra
4. BinBase : The processed data were processed, in-house programmed database built for metabolite identification.

Statistical analysis

StatSoft STATISTICA · TM4 MICROARRAY SOFTWARE SUITE · R · WebGestalt · G2SBC

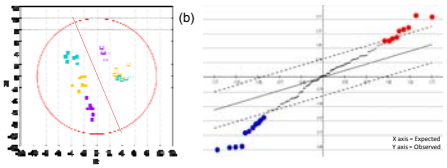
Results

A. Confirmation of Gene X expression and cell growth pattern

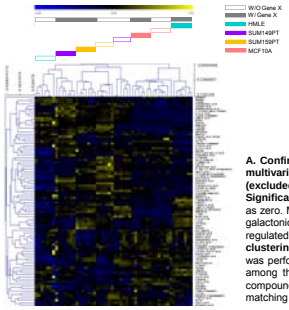


Transcript_ID	Fold change (OE)	Fold change (KD)
NM_177524	2.12	13.35

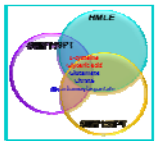
B. Multivariate statistics (Metabolite profiles)



C. Hierarchical clustering analysis

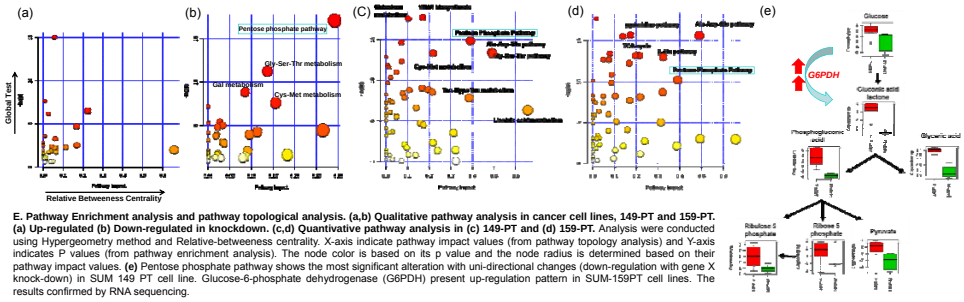


D. Univariate Statistics – The metabolites significantly altered with GeneX expression



A. Confirmation of Gene X expression (a) Western blot (b) RNA sequencing (c) Cell growth pattern with or without Gene X. B. Supervised multivariate statistics using partial least square (PLS) analysis of GC-TOF MS data (a) All genotype devided by with or without Gene X (excluded MCF10A). Differential metabolic phenotypes were detected according to cell lines and genotypes. Vector1 (t1) separate genotypes. **(b) Significance analysis of microarray (SAM)** were performed for screening genotype-specific metabolite alteration. False discovery rate was maintained as zero. Metabolites located up-right (red circle) indicates significantly up-regulated ones with existence of gene X (ornithine, beta-hydroxyl butyric acid, galactonic acid, 1-monopalmitin, isoleucine, uracil, L-cysteine, and arabitol) while compounds of bottom-left (blue circle) present significantly down-regulated ones (citrate, malate, fumarate, N-carbamoylaspartate, glutamate, alanine, threonine, and linoleic acid). **C. Heatmap using Hierarchical clustering analysis (HCL).** Results demonstrated chemical/biochemical clusters according to cell lines and partially genotype (n = 6 or 7). The analysis was performed using Kendall's Tau distance matrix and average linkage. **D. (a) Univariate statistics using t-test.** 5 compounds are common factors among the three groups. L-Cysteine and glyceric acid were up-regulated and glutamate, citrate, and N-carbamoylaspartate were down-regulated compounds. **(b) Modified schematic pathway for pyrimidine synthesis (de novo)** and box and whisker plots indicate up- or down-pattern that matching compounds. There is close correlation with mTOR activation.

E. Qualitative and quantitative Pathway Enrichment Analysis using metabolites in SUM-PT cell lines

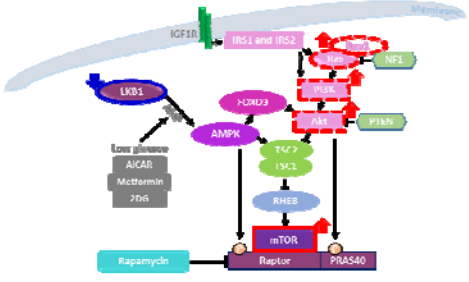


E. Pathway Enrichment analysis and pathway topological analysis. (a,b) Qualitative pathway analysis in cancer cell lines, 149-PT and 159-PT. (a) Up-regulated (b) Down-regulated in knockdown. (c,d) Quantitative pathway analysis in (c) 149-PT and (d) 159-PT. Analysis were conducted using Hypergeometry method and Relative-betweeness centrality. X-axis indicate pathway impact values (from pathway topology analysis) and Y-axis indicates P values (from pathway enrichment analysis). The node color is based on its p value and the node radius is determined based on their pathway impact values. **(e)** Pentose phosphate pathway shows the most significant alteration with uni-directional changes (down-regulation with gene X knock-down) in SUM 149 PT cell line. Glucose-6-phosphate dehydrogenase (G6PDH) present up-regulation pattern in SUM-159PT cell lines. The results confirmed by RNA sequencing.

F. Pathway Enrichment Analysis using RNAseq data

Table 1. Top10 pathways modulated with Gene X expression

Pathway Name	# Gene
Arf6 downstream pathway	45
Glypican pathway	48
mTOR signaling pathway	45
Thrombin/protease-activated receptor (PAR)	45
EGFR-dependent Endothelin signaling events	45
Insulin Pathway	45
Internalization of ErbB1	45
Syndecan-1-mediated signaling events	45
Proteoglycan syndecan-mediated signaling events	46
PAR1-mediated thrombin signaling events	45



G. Common molecular alteration in breast cancer

Table 2 The altered genes with breast cancer-specificity

Pathway Name	Gene list	Score(%)
Pattern 1 : HMLE_OE ▲, 159PT_KD ▼: 25 genes		
Expression : Up	PKM2 ATF4 GGCX	13.0
Expression : Up, Down	RSU1 IP6KW	8.70
Pattern 2 : HMLE_OE ▼, 159PT_KD ▲: 12 genes		
Expression : Up, Down	ABLIM3 RRBP1	18.2

Table 3 The genes modified with general cancers

Gene	Reference
SNORA3	Lung cancer
G6PD	***Strong association with tumor***
PRKCSH	Protein kinase C substrate 80K-H
RSU1	RAS suppressor gene: defense???
ANTXR2	Identified as a result of up-regulation during capillary morpho genesis of endothelial cells (ECs) cultured in vitro
TSPAN4	Overexpressed in hepatocellular carcinoma and accelerates tumor cell growth
MBD3	Tumor suppressor gene

F. Pathway common analysis using overlapped 790 genes between up-regulation with Gene X in normal cell line and down-regulation with Gene X in cancer cell line. The results display in diagram form (modified schematization of *Nature Reviews Cancer*). Line and dotted lines accord closely with the results of pathway common and KEGG. **G. Common molecular alterated genes that concident with 790 genes (Pattern 1) and opposite expression condition (Pattern 2).** PKM2 : Pyruvate kinase, ATF4 : Activating transcription factor 4, GGCX : gamma-glutamyl carboxylase, RSU1 : Ras suppressor protein1, IP6K2 : Inositol hexakisphosphate kinase 2, ABLIM3 : Actin binding LIM protein family, member 3, RRBP1 : Ribosomal binding protein 1 homolog 180kDa. (**Table 2**) The lists is up-regulation with gene X (**pattern 1**) among the genes modified with general cancers (**Table 3**).

Conclusions

1. Unique metabolite profiling of 4 different cell lines and 2 different genotypes (gene X) using GC-TOF/MS data analysis
2. Precise capture of genotype-specific metabolite alteration across different cell lines by intergrative analysis between metabolites and mRNA seq
3. Proposal of detailed mechanistics of biochemical responses to gene X (knock-down of the gene in breast cancer cell lines)
4. Discovery of putative endogenous substrate of the gene

References

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2. Issam Ben-Sahra et al (2013) Stimulation of de Novo Pyrimidine Synthesis by Growth Signaling Through mTOR and S6K1, *Science*
3. Tao Hu et al (2013) Variant G6PD levels promote tumor cell proliferation or apoptosis via the STAT3/5 pathway in the human melanoma xenograft mouse model, *BMC Cancer*