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Identifying crosstalk of mTOR signaling pathway of lobular breast carcinomas

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Abstract. – BACKGROUND: Invasive lobular carcinoma (ILC) and its variants represent 5% to 15% of all invasive breast cancers diagnoses annually. AS a serine/threonine kinase, mammalian target of rapamycin (mTOR) is often a downstream effector of PI3K/Akt (phosphatidyl inositol 3-kinase/protein kinase B) signaling pathway in breasts and many types of cancer cells. Therefore, agents that target mTOR in direct or indirect manner are being developed in anti-cancer therapy.

AIM: In this study, our objective here was to explore more crosstalk pathway with mTOR signaling pathway.

MATERIALS AND METHODS: We collected pathways data from published database, then based on bioinformatics methods we analyzed the significant pathways in the database, additionally, the crosstalk pathways were also analyzed which were defined as those pathways which have the overlapping genes with each other.

RESULTS: As we expected, the results showed that Notch signaling pathway (hsa04330), Regulation of autophagy (hsa04140), and Adipocytokine signaling pathway (hsa04920) were linked to mTOR signaling pathway. All of them have been demonstrated participate in breast cancer progression.

CONCLUSIONS: We obtained some key pathways that crosstalked with mTOR signaling pathway, we hope our study could provide novel therapeutic approaches for breast cancer.

Key Words:
Invasive lobular carcinoma, mTOR signaling pathway, Pathway crosstalk.

Introduction

Breast cancer is the second-most common cause of cancer-related death in women. Invasive lobular carcinoma (ILC) and its variants represent 5% to 15% of all invasive breast cancers diagnoses annually. Usually, the tumors cells are estrogen receptor (ER) and progesterone receptor (PR) positive, without over-expression or amplification of the HER-2/neu gene (human epidermal growth factor-receptor2/neu gene)[1,2]. Thereby, the increased use of antiestrogen, such as tamoxifen, may have attenuated ILC incidence. However, more anti-cancer drug is imminent[3].

Mammalian target of rapamycin (mTOR) is a 289-kDa serine/threonine kinase that is often a downstream effector of PI3K/Akt (phosphatidyl inositol 3-kinase/protein kinase B) signaling pathway in breasts and many types of cancer cells. mTOR functions as two distinct multiprotein complexes, mTORC1 and mTORC2. In the presence of mitogenic stimuli and sufficient nutrients and energy, mTORC1 phosphorylates p70 S6 kinase (S6K1) and eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1). The S6Ks have been implicated in the translational regulation of mRNAs that typically encode ribosomal proteins as well as components of the translational machinery. The mTOR-dependent phosphorylation of 4E-BP1 mediates its dissociation from the RNA cap-binding protein eIF-4E, thereby allowing reconstitution of a translationally competent initiation factor complex (eIF-4F). In contrast, mTORC2 is insensitive to nutrients or energy conditions. In response to hormones or growth factors, mTORC2 phosphorylates Akt, and regulates actin cytoskeleton and cell survival[4,5].

The importance of mTOR signaling in cancer progression is now widely accepted. Consequently, a number of agents that selectively target mTOR are being developed in anti-cancer therapy, such as rapamycin and its analogues such as CCI-779 and RAD001. Pre-clinical studies that used breast cancer cell lines have suggested that p-Akt or p-S6K1 expressing tumors, as well as phosphatase and tensin homolog (PTEN) negative tumors, were sensitive to rapamycin[6]. RAD001 (everolimus) is an orally bioavailable, mTOR inhibitor currently in phase II clinical trials in cancer patients. Irrespective of estrogen receptor (ER) status, breast cancer cell lines seem particu-

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larly sensitive to RAD001, with IC50 values for *in vitro* antiproliferative activity in the sub- to low nanomolar range[7]. RAD001 attenuates radiation-induced prosurvival Akt/ mTOR signaling and enhances the cytotoxic effects of radiation in breast cancer cell models, showing promise as a method of radiosensitization of breast cancer[8]. In addition, mTOR has also been implicated as one of the target of phospholipase D (PLD) in breast cancer to promote survival. Study found that elevated PLD activity in the MDA-MB-231 human breast cancer cell line generates an mTOR-dependent survival signal that is independent of PI3K, indicating an alternative survival signal that is dependent on PLD and mTOR and is active in a breast cancer cell line where the PI3K survival pathway is not active[9].

In this study, we performed the research on the PPI-interaction network, significant pathway, and crosstalk between pathways based on the previous microarray analysis[10]. Identically, we demonstrated mTOR signaling pathway was a significant pathway involved in ILC progression. A number of crosstalk pathways with mTOR signaling pathway were further mined, with hope to suggest novel therapeutic approaches for breast cancer.

Materials and Methods

Data Sources

We download all the pathways from Kyoto encyclopedia of genes and genomes (KEGG)[11] and protein-protein interaction (PPI) datasets from human protein reference database (HPRD)[12] and bio general repository for interaction database (BIOGRID)[13] database.

Then construct an ensemble protein-protein interaction network by integrating two above existing PPI databases in human. Total 326119 unique PPI pairs were collected in which 39240 pairs are from HPRD and 379426 pairs are from BIOGRID.

We extracted the gene expression profile GSE5764[14] data on lobular breast carcinomas (ILC) with normal tissues, which were deposited in National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) (http://www.ncbi.nlm.nih.gov/geo/) database. Tumor and normal tissues from the same mammary gland were identified by an experienced pathologist, snap-frozen in liquid nitrogen and stored at -80ºC for further analysis. The gene expression profiles of cortical tubers were compared with autopsy control specimens and perituberal tissue from the same patients to study the significant expressed pathways and connection among them in the invasive lobular carcinoma (ILC).

The limma method[15] was used to identify differentially expressed genes (DEGs). The original expression datasets from all conditions were processed into expression estimates using the robust multiarray overaging (RMA) method with the default settings implemented in Bioconductor, and then construct the linear model. The DEGs only with the fold change > 2 and p-value < 0.05 were selected.

Significant Pathways Analysis

We adopted an impact analysis that includes the statistical significance of the set of pathway genes but also considers other crucial factors such as the magnitude of each gene's expression change, the topology of the signaling pathway, their interactions, etc[16]. In this model, the Impact Factor (IF) of a pathway Pi is calculated as the sum of two terms:

$$IF\ (\mathrm{Pi}) = \log\left(\frac{1}{\mathrm{pi}}\right) + \frac{\sum_{\mathrm{g}\in \mathrm{Pi}} |\mathrm{PF}|\ (\mathrm{g})}{|\mathrm{VE}| \cdot N_{de}\ (\mathrm{Pi})}$$

The first term is a probabilistic term that captures the significance of the given pathway P_i from the perspective of the set of genes contained in it.

It is obtained by using the hyper geometric model in which p_i is the probability of obtaining at least the observed number of differentially expressed gene, N_{de}, just by chance[17,18].

The second term is a functional term that depends on the identity of the specific genes that are differentially expressed as well as on the interactions described by the pathway (i.e., its topology).

The second term sums up the absolute values of the perturbation factors (PFs) for all genes g on the given pathway P_i.

The PF of a gene g is calculated as follows:

$$PF(g) = VE(g) + \sum_{u \in USg} \beta_{ug} \cdot \frac{PF(u)}{N_{ds}(u)}$$

In this equation, the first term $E\ (g)$ captures the quantitative information measured in the gene expression experiment. The factor $E\ (g)$ repre-

sents the normalized measured expression change of the gene g. The first term $E(g)$ in the above equation is a sum of all PFs of the genes u directly upstream of the target gene g, normalized by the number of downstream genes of each such gene $N_{ds}(u)$, and weighted by a factor β_{ug}, which reflects the type of interaction: $\beta_{ug} = 1$ for induction, $\beta_{ug} = -1$ for repression (KEGG supply this information about the type of interaction of two genes in the description of the pathway topology). US_g is the set of all such genes upstream of g. We need to normalize with respect to the size of the pathway by dividing the total perturbation by the number of differentially expressed genes on the given pathway, $N_{de}(P_i)$. In order to make the *IF*s as independent as possible from the technology, and also comparable between problems, we also divide the second term in the first equation by the mean absolute fold change E, calculated across all differentially expressed genes.

Pathway Crosstalk Analysis

Here the crosstalk pathways are defined as those pathways which have the overlapping genes and edges with each other. The overlapping genes mean both of the two pathways included and the overlapping edges mean both of the two pathways included the PPI interaction edges.

To determine the co-expressed significance of a gene pair in disease cases, we used the PCC test to calculate the p-value.

Map those p-values to the nodes and edges in the PPI network collected from the HPRD[12] and BIOGRID[13] database. The following formula is used to define a function as the combination of statistical significance of an interaction by a scoring scheme. The detail desprcition could be seen in Liu et al[19].

$$S(e) = \mathrm{f}\,[diff\,(x), corr\,(\mathrm{x}, \mathrm{y}), diff\,(y)]$$

$$= -2 \sum_{\mathrm{i}=1}^{\mathrm{k}} \log_e(pi)$$

The diff (x) and diff (y) are differential expression assessments of gene x and gene y, respectively. corr (x,y) represents their correlation between gene x and gene y. f is a general data integration method that can handle multiple data sources differing in statistical power. Where k = 3, p1 and p2 are the p-values of differential expression of two nodes, p3 is the p-value of their co-expression.

Pathway Crosstalk Analysis

The detailed analysis of crosstalk of relationships among pathways is then investigated, especially that with overlap of two significant pathway analysis results.

To define the interaction significance between pathways, we summarize all the scores of edges S(e) of all non-empty overlaps. Specifically, the interaction score between two pathways is estimated by their overlapping status of weighted pathways in the following formula:

$$C(pi, pj) = \sum_{e \in \mathrm{Oij}} S(e)$$

where Pi and Pj are two pathways, and O is their overlapping.

To estimate the significance of the overlapping between different pathways, we random sample 10^5 times of the same size two pathways in the edges of pathway network and calculate their overlapping scores. The frequency larger than C is regarded as the interaction significance p-value. We considered as the significant crosstalks with the p-value < 0.01.

Significant GO Enrichment Analysis in Each Pathway

The functional enrichment among proteins in one pathway is defined as:

$$P = 1 - \sum_{\mathrm{i}=0}^{\mathrm{k}-1} \frac{\binom{f}{i}\binom{n-f}{m-i}}{\binom{\mathrm{n}}{\mathrm{m}}}$$

where n is the number of nodes in the network, f is the number of proteins annotated with a particular gene ontology (GO) function, m is the number of proteins involved in the pathway and k is the frequency of the GOID. We identified the GO function enrichment of the pathways respectively with the p-value < 0.05.

Results

To get DEGs of lobular breast carcinomas (ILC), we obtained publicly available microarray data sets GSE5764 from GEO. After microarray analysis, the differentially expressed genes with the fold change > 2 and p-value < 0.05 were selected. 797 genes were selected as DEGs from GSE5764.

Significant Pathway in ILC

To identify the relevant pathways changed in ILC, we used a statistical approach on pathway level. Significance analysis at single gene level may suffer from the limited number of samples and experimental noise that can severely limit the power of the chosen statistical test. Pathway can provide an alternative way to relax the significance threshold applied to single genes and may lead to a better biological interpretation. So, we adopted a pathway based impact analysis method that contained many factor including the statistical significance of the set of differentially expressed genes in the pathway, the magnitude of each gene's expression change, the topology of the signaling pathway, their interactions and so on. The impact analysis method yielded many significant pathways containing Leukocyte transendothelial migration, Cell adhesion molecules (CAMs), Adherens junction, ECM-receptor interaction, mTOR signaling pathway and so on (Table I only list top 10 of significant pathways).

Pathway Crosstalk Between Pathways

We considered the pathway crosstalk between mTOR signaling pathway (hsa04150) and other pathways detected by the overlaping score. We found that these 8 pathways crosstalk with the mTOR signaling pathways (Figure 1). The Adipocytokine signaling pathway (hsa04920), Notch signaling pathway (hsa04330), Wnt signaling pathway(hsa04310) crosstalk to the mTOR signaling pathway (hsa04150) with the p-value < 0.01, though the top 10 of significant pathways crosstalk with mTOR signaling pathway (hsa04150) with the p-value > 0.01 (not display in the Figure 1).

Crosstalk of GO Relationships Among Pathways

For detail analysis the crosstalk between the pathways, use the hypergeometric test to find the significant GOID in each pathway with the p-value < 0.05, respectively. The results of the top five GOID in part of the pathways are use to construct the connection among pathways. From the significant GO enrichments, we know the crosstalk of GO biological processes during the disease development among the pathways. In the Figure 1, we only found 3 relationships crosstalk through the significant GOID. The mTOR signaling pathway (hsa04150) crosstalk with Notch signaling pathway (hsa04330) through the gene expression (GO: 0010467) and crosstalk with Adipocytokine signaling pathway (hsa04920) through the regulation of fatty acid oxidation (GO: 0046320) and also crosstalk with Regulation of autophagy (hsa04140) through innate immune response (GO: 0045087), and cell cycle (GO: 0007049).

Discussion

Using invasive lobular carcinomas and normal tissues microarray specimens, we identified a number of pathways associated with ILC progression, such as Leukocyte transendothelial migration, Cell adhesion molecules (CAMs), Adherens junction, extracellular matrix-receptor (ECM-receptor) interaction, mTOR signaling pathway and so on. Several lines of evidence have demonstrated that defects in E-cadherin (E-CD) is a distinguishing feature in ILC resulting in decreased cell-cell adhesiveness and increased tumor metastasis[20]. Abnormal cytoplasmic and

Table I. Significant pathway analysis result.

Database name	Pathway name	Impact factor	% pathway genes in input	Corrected gamma p-value
KEGG	Leukocyte trans endothelial migration	311.986	5.042	1.00E-133
KEGG	Cell adhesion molecules (CAMs)	307.516	3.731	8.65E-132
KEGG	Adherens junction	39.85	7.692	2.02E-16
KEGG	ECM-receptor interaction	17.669	19.048	3.96E-07
KEGG	Focal adhesion	16.807	11.823	8.94E-07
KEGG	Circadian rhythm	16.331	15.385	1.40E-06
KEGG	Acute myeloid leukemia	7.861	11.864	0.003415811
KEGG	mTOR signaling pathway	6.719	1.923	0.009322586
KEGG	Type 2 diabetes mellitus	6.703	2.222	0.009453312
KEGG	Melanoma	6.607	8.451	0.010276134

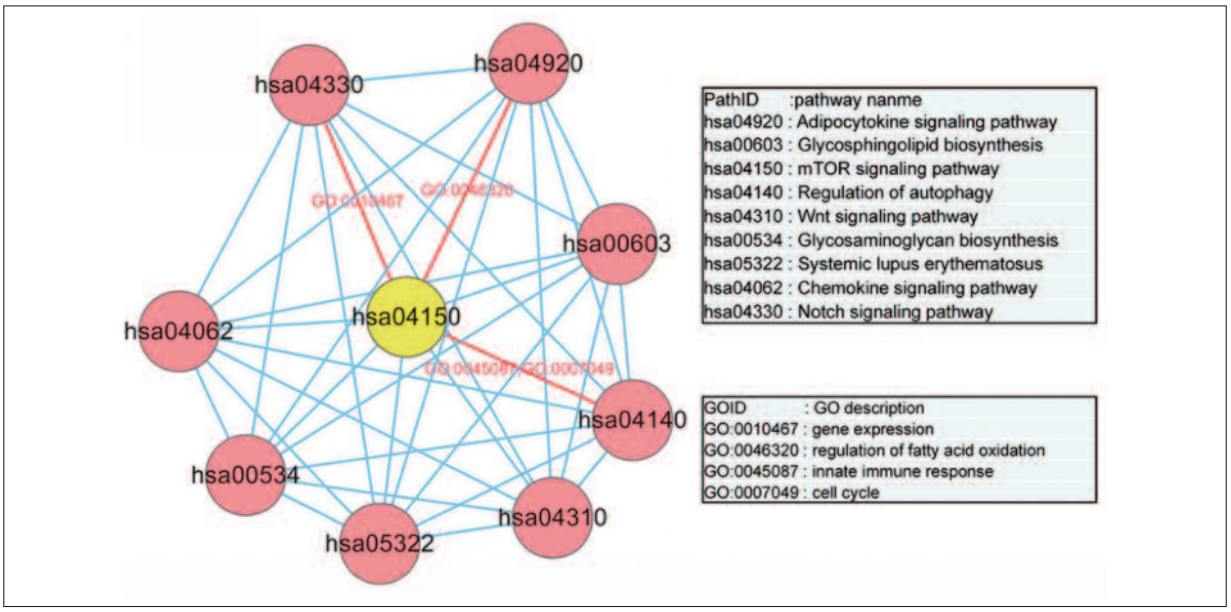

Figure 1. Crosstalk between mTOR signaling pathway (hsa04150) and other pathways. Red line means the two pathways connected with the same GOID; the blue line means any two pathways crosstalk with the p-value < 0.01. The right two tables mean the description of the pathway and GOID.

nuclear localization of p120, which are mediated by the absence of extracellular domain (E-CD), characteristically occur in the early stages of lobular breast cancer and are maintained during tumor progression to metastasis. Consequently, p120 may be an important mediator of the oncogenic effects derived from E-CD inactivation, including enhanced motility and invasion, in lobular breast cancer[21]. Matrix metalloproteinases (MMP)-11 has been identified over-expressed in many ILC cells and that it may play a role in lobular carcinogenesis through increasing resistance to anoikis, a programmed cell death triggered by a lack of proper cell matrix interaction[22].

However, importantly, our study was paid more attention to mTOR signaling pathway. Therefore, 8 pathways were found crosstalk with the mTOR signaling pathways in further analysis. Of them, the Adipocytokine signaling pathway, Notch signaling pathway, Wnt signaling pathway were found crosstalk to the mTOR signaling pathway with the p-value < 0.01. The Adipocytokine signaling pathway (hsa04920), Notch signaling pathway, and Regulation of autophagy were found crosstalk with the mTOR signaling pathway in GO analysis. In a word, our suggested a crosstalk was present between the Adipocytokine signaling pathway, Notch signaling pathway, Wnt signaling pathway, Regulation of autophagy, and mTOR signaling pathway.

From the KEGG pathway, we could found a crosstalk is present between Adipocytokine signaling pathway and mTOR signaling pathway. Interaction between insulin receptor substrate 1(IRS1) and mTOR has been demonstrated in MCF-7 breast cancer cell. IRS-1 is an adaptor protein important for insulin and IGF-I receptor (Insulin-like Growth Factor-IR) transduction to downstream targets. One mechanism recently identified to down-regulate IGF-I or insulin receptor signaling in diabetic models is IRS-1 Ser312 phosphorylation. IGF-I treatment converge downstream onto mTOR to induce IRS-1 Ser312 phosphorylation[23]. Besides, adiponectin-repressed proliferation in breast cancer cells is mediated through an inactivation of p44/42 MAPK (mitogen-activated-protein-kinase) protein 1 and 3 expression, a stimulation of adenosine monophosphate-activated protein kinase (AMPK) activity by phosphorylation at Thr172 and a decrease in Akt phosphorylation (Thr308) associated with an increased expression of liver kinase B1 (LKB1) leading to a reduction of mTOR activity as evidenced by reduced phosphorylation of S6K[24]. Leptin also induces the PI3K/Akt/mTOR survival pathway by activating the phosphorylation of Akt Thr308 or Akt Ser473 and by stimulating the protein expression of protein kinase C-alpha (PKC-alpha), which is controlled by PI3K[25].

mTOR signaling has been reported to crosstalk with the Notch signaling pathway in several malignant cells[26]. Cells expressing intracellular domain of human Notch1 (NIC-1) are chemoresistant in a wild-type p53-dependent manner. Inhibition of p53 by NIC-1 mainly occurs through mTOR using PI3K-Akt/protein kinase B (PKB) pathway as the mTOR inhibitor, rapamycin treatment abrogated NIC-1 inhibition of p53 and reversed the chemoresistance. Further, ectopic expression of eIF4E inhibited p53-induced apoptosis and conferred protection against p53-mediated cytotoxicity to similar extent as that of NIC-1 overexpression. MCF7 (breast cancer) has aberrant Notch1 signaling that can be reversed by both PI3K and mTOR inhibitors[27]. Inhibition of the Notch pathway with a ³-secretase inhibitor (GSI) was found decreased both the Notch and the mammalian target of rapamycin/AKT pathways. The AKT/mTOR signaling pathway is known to influence cell growth through anabolic processes that control glucose metabolism, ATP production, and glucose uptake through Hypoxia-inducible Factor-1 (HIF1) transcriptional control of the glucose transporter GLUT1. Antitumor activity resulting from GSI treatment was associated with decreased expression of glucose transporter Glut1[28].

Autophagy is an alternative cell death pathway when apoptosis is defective. Autophagy is found to be suppressed in malignant tumors and involved in tumorigenesis. A number of studies have reported that autophagy is activated in response to various anticancer therapies and regulated through PI3K/AKT/mTOR pathway[29]. Such as plumbagin exhibited cell proliferation inhibition by inducing cells to undergo G2-M arrest and autophagic cell death. Plumbagin inhibited survival signaling through the phosphatidylinositol 3-kinase/AKT signaling pathway by blocking the activation of AKT and downstream targets. Phosphorylation of both of mammalian target of rapamycin downstream targets, p70 ribosomal protein S6 kinase and 4E-BP1, was also diminished. Overexpression of AKT by AKT cDNA transfection decreased plumbagin-mediated autophagic cell death, whereas reduction of AKT expression by small interfering RNA potentiated the effect of plumbagin, supporting the inhibition of AKT being beneficial to autophagy[30].

Although our analysis indicated there was a crosstalk between Wnt signaling pathway and mTOR signaling pathway, direct evidence is still scarce. We suggested an indirect manner was present between them mediated by Notch signaling[31] or Autophagy[32,33].

In this study, a network-based approach was used to analyze the crosstalk with mTOR signaling pathway. The results showed mTOR signaling pathway was high related with the Notch signaling pathway (hsa04330), Regulation of autophagy (hsa04140) and Adipocytokine signaling pathway (hsa04920), indicating cooperation in response to ILC. The results are all in consistent with our prior knowledge. The crosstalk of pathways may present new novel therapeutic approaches for ILC. In addition, our work showed that comprehensive and system-wide analysis provides evidence for ILC and complements the traditional component-based approaches.

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