RESEARCH ARTICLE

Protein-protein Interaction Network Analyses for Elucidating the Roles of LOXL2-delta72 in Esophageal Squamous Cell Carcinoma

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Abstract

Lysyl oxidase-like 2 (LOXL2), a member of the lysyl oxidase (LOX) family, is a copper-dependent enzyme that catalyzes oxidative deamination of lysine residues on protein substrates. LOXL2 was found to be overexpressed in esophageal squamous cell carcinoma (ESCC) in our previous research. We later identified a LOXL2 splicing variant LOXL2-delta72 and we overexpressed LOXL2-delta72 and its wild type counterpart in ESCC cells following microarray analyses. First, the differentially expressed genes (DEGs) of LOXL2 and LOXL2-delta72 compared to empty plasmid were applied to generate protein-protein interaction (PPI) sub-networks. Comparison of these two sub-networks showed hundreds of different proteins. To reveal the potential specific roles of LOXL2-delta72 compared to its wild type, the DEGs of LOXL2-delta72 vs LOXL2 were also applied to construct a PPI sub-network which was annotated by Gene Ontology. The functional annotation map indicated the third PPI sub-network involved hundreds of GO terms, such as "cell cycle arrest", "G1/S transition of mitotic cell cycle", "interphase", "cell-matrix adhesion" and "cell-substrate adhesion", as well as significant "immunity" related terms, such as "innate immune response", "regulation of defense response" and "Toll signaling pathway". These results provide important clues for experimental identification of the specific biological roles and molecular mechanisms of LOXL2-delta72. This study also provided a work flow to test the different roles of a splicing variant with high-throughput data.

Keywords: LOXL2-delta72 - protein-protein interaction - network comparison - esophageal SCC

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Introduction

Lysyl oxidase-like 2 (LOXL2) is a member of the lysyl oxidase (LOX) family which is composed of five homologs (LOX and LOXL1-4). They are copper-dependent enzymes that catalyze oxidative deamination of lysine residue of their protein substrats (Kim et al., 1995). It has been found LOXL2 is overexpressed and plays a crucial role in the metastasis of various malignancies, such as colon cancer, cholangiocarcinoma, pancreatic carcinoma (Fong et al., 2007; Gao et al., 2008; Rückert et al., 2010). Ovexpression of LOXL2 negatively influences the survival of breast cancer patients (Ahn et al., 2013). The extracellular matrix remodeling mediated by the secreted LOXL2 is supposed to be responsible for promoting the invasion of breast cancer cells through the upregulation of tissue inhibitor of metalloproteinase-1 (TIMP-1) and matrix metalloproteinase-9 (MMP-9) (Barker et al., 2011). Moreover, increased LOXL2 could promote gastric tumor invasion and metastases via the Src kinase/Focal adhesion kinase (Src/FAK) pathway (Peng et al., 2009). Evidences suggested that intracellular LOXL2 mediates the induction of epithelial–mesenchymal transition (EMT) by stabilizing Snail through the oxidization of its lysine residues K98 and K137 to repress the expression of E-cadherin, which also indicates a contribution of LOXL2 to tumor progression (Peinado et al., 2005). Alternatively, LOXL2 is able to downregulate E-cadherin through the deamination of the Lys4 of H3K4 (me3), which also reveals LOXL2 might contribute to tumorigenesis by affecting other key genes as a H3K4 modulator (Herranz et al., 2012).

Our previous research found LOXL2 was overexpressed in esophageal squamous cell carcinoma (ESCC), which was associated with lymph node metastasis. LOXL2 protein manifested decreased nuclear expression and increased cytoplasmic expression. Overall survival rates of the ESCC patients with decreased nuclear expression or increased cytoplasmic expression of LOXL2 were

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significantly lower than those of the patients with the reverse expression pattern. The nuclear expression of LOXL2 was an independent prognostic factor for ESCC (Li et al., 2012). Recently, we identified a splicing variant of LOXL2 with the loss of 24 amino acids in its exon 5 and we name it LOXL-delta72. LOXL-delta72 was also elevated in ESCC cell lines and clinical samples compared to the control, indicating it might play important roles in ESCC (data prepared in another manuscript of our lab). To reveal its biological roles and molecular mechanism, we overexpressed LOXL2 wild type (LOXL2WT) and LOXL2-delta72 in ESCC cell line KYSE150 and the mRNA expression profiles were analyzed by PrimeView™ Human Gene Expression Array (Affymetrix Corp., St Clara, CA, USA).

Protein-protein interactions (PPI) are critical for most cellular processes and the multi-functionality of a single protein. Given the rapidly growing numbers of public PPI data, analyses of PPI networks is important for inferring the function of proteins and has become a major thrust in systems biology research (Zhu et al., 2007). In the recent years, the integration analyses of gene expression data with PPI network have received considerable attention to mine the biological meanings (Lee et al., 2009; Li et al., 2012). Zhu et al. applied a weighted gene co-expression network to identification prognosis markers in endometrial cancer for potential therapeutic targets (Zhu et al., 2012).

In this study, two PPI sub-networks were generated by mapping the differentially expressed genes (DEGs) from the LOXL2WT and LOXL2-delta72 overexpression microarray results, respectively. These two PPI sub-network were analyzed and compared. To reveal the specific influence of LOXL2-delta72, a third PPI sub-network was constructed by the DEGs from the comparison of LOXL2WT and LOXL2-delta72 overexpression results, which was annotated by Gene Ontology. These analyses provided important clues to identify the specific roles of LOXL2-delta72 from the view of system analysis.

Materials and Methods

The differentially expressed genes

The detailed manipulations of overexpression and microarray were introduced in another manuscript from our lab. Briefly, LOXL2WT and LOXL2-delta72 were ovexexpressed using pcDNA3.1 plasmid in ESCC KYSE150 cells with an empty plasmid as a control. The total RNA was extracted by TRIzol and analyzed by PrimeView™ Human Gene Expression Array (Affymetrix Corp., St Clara, CA, USA). The raw data were normalized by RMA algorithm. These mRNA expression profiles have been submitted to GEO database (http://www.ncbi.nlm.nih.gov/geo/) with accession number of GSE53645 (Supplementary file 1). The differentially expressed genes (DEGs) of LOXL2WT vs empty plasmid and LOXL2-delta72 vs empty plasmid were obtained by the traditional 2-fold change. The DEGs of LOXL2-delta72 vs LOXL2WT were obtained using the the threshold of 1.5-fold change.

PPI sub-networks generation

Human Protein Reference Database (HPRD) (http://www.hprd.org/) is an object database that integrates a wealth of information relevant to the function of human proteins in health and disease including protein features, post-translational modifications (PTMs) and protein–protein interactions (Keshava et al., 2009). The Biological General Repository for Interaction Datasets (BioGRID) (http://thebiogrid.org/) is a public database that archives and disseminates genetic and protein interaction data from model organisms (Chatr-Aryamontri et al., 2013). All of these PPI data are manually collected from literatures of experimentally validation, which have also been widely applied in human PPI network research in the post "omics" era because of their reliability (Lehne et al., 2009; Koh et al., 2012).

In this study, only the newest version PPI data of Homo sapiens species were downloaded from these two databases and integrated by removing the redundant interactions. The integrated PPI data containing 18595 unique proteins and 174552 interactions are used as the parent PPI network. First, three PPI networks were generated by mapping the DEGs of LOXL2WT vs empty plasmid (LOXL2WT-DEGs), LOXL2-delta72 vs empty plasmid (LOXL2-delta72-DEGs) and LOXL2-delta72 vs LOXL2WT (delta72-WT-DEGs) to the HPRD&BioGRID parent PPI network by applying the Cytoscape software, respectively. Cytoscape (http://www.cytoscape.org/) is an open source software with various plugins for manipulating biomolecular interaction networks with expression data or other molecular states into a unified conceptual framework. Cytoscape is most powerful in the query, visualization and manipulation of high-throughput data of protein-protein, protein-DNA, and genetic interactions (Cline et al., 2007). In Cytoscape, each protein is represented as a node, and each interaction between two nodes is represented as an edge. To increase the liabilities and define the protein perturbation to a certain level, the network reconstruction was limited to the first protein neighbors of these DEGs.

PPI sub-networks topological parameters analyses

Network theory provides a quantifiable description for networks and there are several network topological parameters that enable the comparison and characterization of complex networks (Pavlopoulos et al., 2011). In order to gain insight into the organization and structure of the PPI network, NetworkAnalyzer was applied to analyze the network topological parameters. NetworkAnalyzer (http://med.bioinf.mpi-inf.mpg.de/netanalyzer/index.php) is able to calculate a number of network topological parameters for directed and undirected networks, such as distributions of node degree, clustering coefficient, network centralization and network density (Assenov et al., 2008).

Power law distribution of node degree, one of most important network topological characteristics, was analyzed as we performed previously (Wu et al., 2013). Briefly, the edges in all networks were treated as undirected. The node degree of one node is the number

Table 1. Topological Parameters of LOXL2-delta72-DEGs and LOXL2-WT-DEGs PPI Sub-network

PPI sub-network	$y = \beta\chi^{a}$	R^2	correlation	clustering coefficient	network centralization	network density
LOXL2-delta72	$y = 182.96\chi^{-1.030}$	0.815	0.707	0.304	0.77	0.03
LOXL2-WT	$y = 209.7\chi^{-0.998}$	0.813	0.775	0.295	0.76	0.029

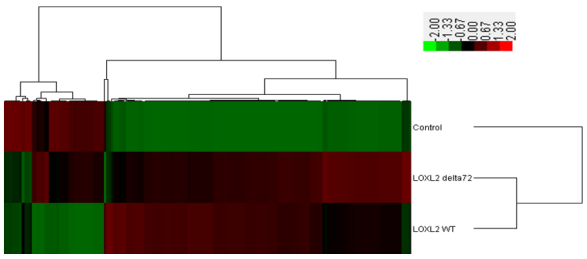

Figure 1. The Cluster of mRNA Expression Profile

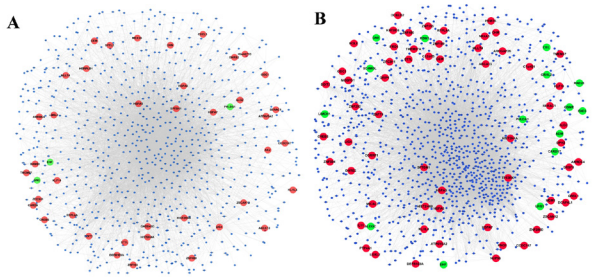

Figure 2. PPI Sub-networks Were Generated by Mapping DEGs to HPRD&BioGRID Parent PPI Network. (A) PPI sub-network of LOXL2-delta72-DEGs. (B) PPI sub-network of LOXL2WT-DEGs. Nodes were labeled by different colors to indicate the expression trend of proteins. Green nodes represented proteins encoded by downregulated genes, while red nodes represented proteins encoded by up-regulated genes. The other interacting proteins without significantly differentially expression were represented as blue nodes

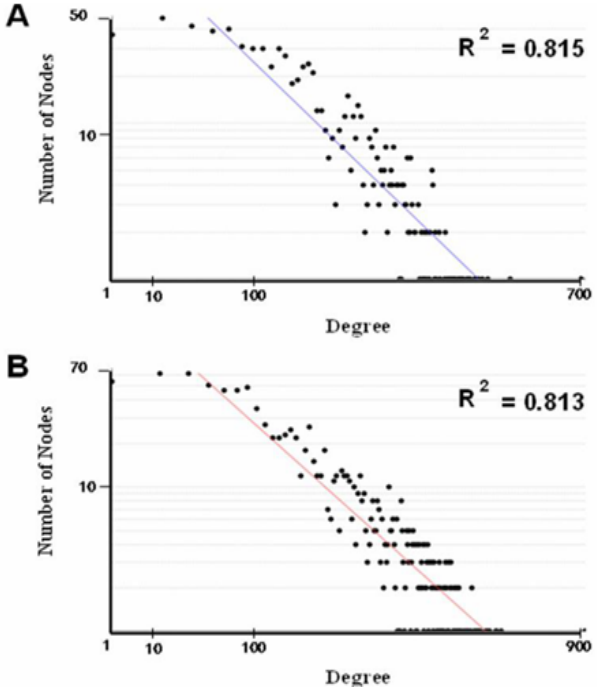

Figure 3. Node-degree Distribution for the PPI Sub-networks. The node degree (k) is represented on the x-axis and the number of nodes with k is represented on the y-axis. The graph displays a decreasing trend of degree distribution with increase in number of links indicating scale free topology. (A) Degree distribution of the PPI sub-network of LOXL2-delta72-DEGs. (B) Degree distribution of the PPI sub-network of LOXL2WT-DEGs

of edges directly linked to it while the node degree distribution in a network offers the number of nodes whose degree is k ($k = 0, 1, \ldots$). Node degree distribution P (k) is the number of nodes with a degree k. By fitting a line on datasets, the pattern of their dependencies can be visualized. NetworkAnalyzer considers only data points with positive coordinate values for fitting the line where the power law curve of the form $y = \beta x^{a}$ is transformed into a linear model $\ln y = \ln \beta + a \ln x$ and the R^2 value (coefficient of determination) provides a measure of how well the data points fit to the curve. If R^2 value is more close to 1, indicating the fit is better.

Functional annotation map generation

To determine whether interacting proteins in the PPI sub-network were clustered according to their molecular function, we integrated the Gene Ontology (GO) "Biological function" terms into the PPI networks by mining the over-represented GO terms of proteins using the ClueGO. ClueGO (http://www.ici.upmc.fr/cluego/) is able to decode and visualize functionally grouped GO terms in the form of networks (Bindea et al., 2009). In this study, over-represented terms with kappa score > 0.3 and P-value < 0.001 were considered significant.

Results

PPI sub-network of DEGs

The expression profile data were log transformed and clustered (Figure 1). Using the traditional two-fold change as threshold, a total of 64 DEGs were obtained including 59 upregulated genes and 5 downregulated genes from the result of LOXL2-delta72 vs empty plasmid. While there were 84 upregulated genes and 22 downregulated genes from the result of LOXL2-WT vs empty plasmid.

In order to understand how many proteins were connected with these DEGs and how the DEGs influence the alternation of cell life, the network of their interactions with other proteins could provide a deep insight into their functions. Two PPI sub-networks were constructed by mapping the LOXL2WT-DEGs and LOXL2-delta72-DEGs to the HPRD&BioGRID parent PPI network, respectively. The LOXL2WT-DEGs PPI sub-network contained 1112 nodes and 17889 edges (interactions), including 60 upreguated DEGs and 15 downregulated DEGs (Figure 2A). The LOXL2-delta72-DEGs PPI sub-network was composed of 818 nodes and 9936 edges, containing 39 upregulated DEGs and 3 downregulated DEGs (Figure 2B). These two PPI sub-networks indicated

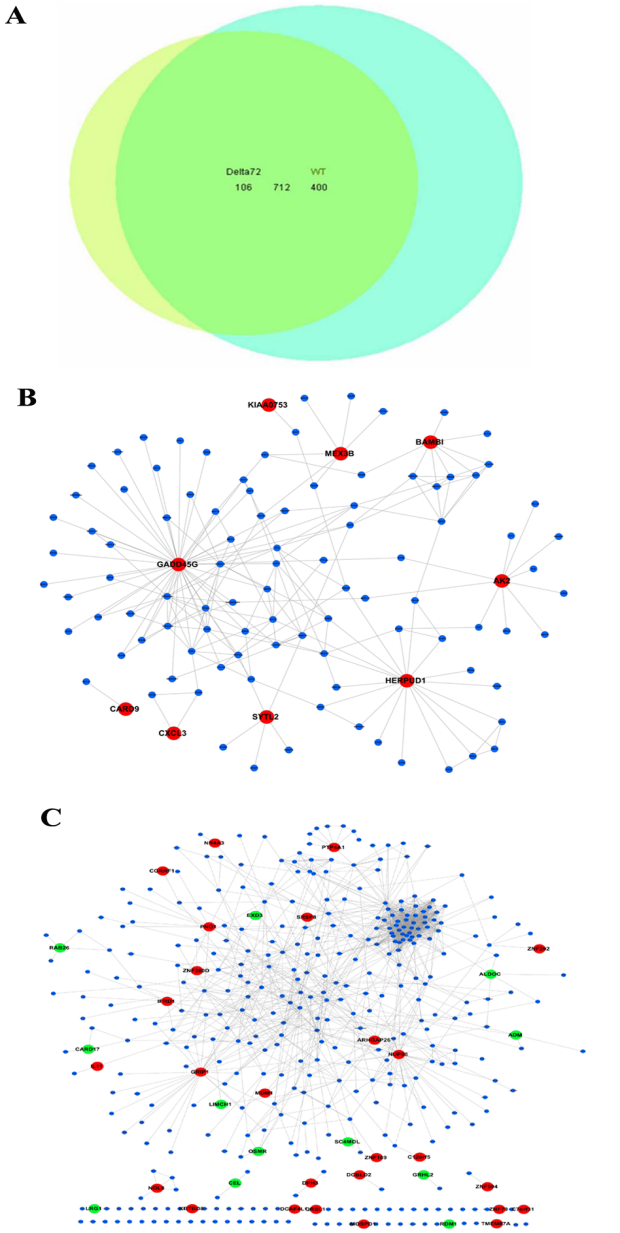

Figure 4. The Comparison of PPI Sub-networks. (A) The number of different nodes of the PPI sub-networks of LOXL2-delta72-DEGs and LOXL2-DEGs compared with each other. (B) The different PPI sub-network of LOXL2-delta72 vs its wild type. (C) The different PPI sub-network of LOXL2 vs LOXL2-delta72

that the overexpression of LOXL2WT or LOXL2-delta72 greatly disturbed the PPI network in ESCC as their DEGs interacts with hundreds and thousands of proteins to enlarge the biological consequences of the protein itself.

Topological parameters of PPI sub-networks

The network topological parameters were analyzed by NetworkAnalyzer. The distributions of node degree followed power law distributions approximately with R^2 = 0.815 and 0.813, respectively (Table 1, Figure 3). Thus, these two PPI sub-networks were scale-free, which is one of the most important characters of true complex biological networks (Barabási et al., 2004). Of other topological parameters of these two sub-networks, such as clustering coefficient, network centralization and network density, were showed in Table 1.

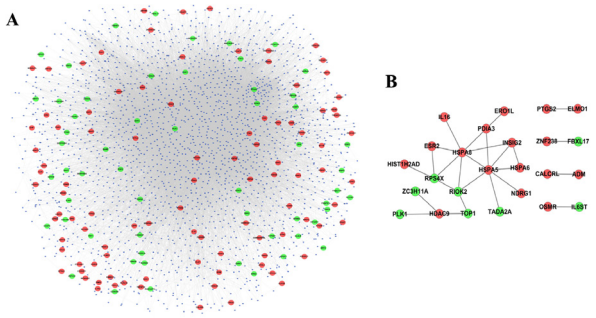

Figure 5. The PPI Sub-network by Mapping the Delta72-WT-DEGs. (A) The PPI sub-networks by mapping delta72-WT-DEGs to HPRD&BioGRID parent PPI network. (B) The internal interactions between the delta72-WT-DEGs

Two PPI sub-networks comparison

We found LOXL2-delta72-DEGs shared greatly the same with LOXL2WT-DEGs when using the 2-fold change, but we presumed the proteins in their PPI sub-network might be diverse as they have multiple different interacting proteins. We applied VennDiagramgenerator program to calculate the LOXL2-delta72-DEGs and LOXL2WT-DEGs PPI sub-networks. As was shown in Figure 4A, there were 712 nodes (proteins) shared by LOXL2-delta72-DEGs and LOXL2WT-DEGs PPI sub-networks, while there were 106 and 400 different nodes (proteins) when compared with each other. To better illustrate the different proteins, we mined them from these two PPI sub-network and also marked the DEGs in the graph. When LOXL2-delta72-DEGs PPI sub-network was compared to LOXL2WT-DEGs PPI sub-network, 9 upregualted DEGs interacting with other 97 proteins involved 207 interactions were differential (Figure 4B). On the other hand, a dozen of DEGs involve 1456 interactions were unique for the LOXL2WT-DEGs PPI sub-network (Figure 4C). This ratio of different proteins was about 1:4, indicating LOXL2-delta72 has a distinguishing influence in ESCC comparing to its wild type as a splicing variant.

Functional annotation map

The functions of a splicing variant might be different and vary compared to its wild type (Blencowe et al., 2006). Accumulated evidences have suggested that splicing abnormalities are a common characteristic of cancer. The potential roles for the alternative splicing in cancer are well documented and include changes in genes associated with cell migration, regulation of cell growth, hormone responsiveness, apoptosis and response to chemotherapy (Wang et al., 2007).

To provide a deep view into the different influence on ESCC cells between LOXL2-delta72 and its wild type, we selected the DEGs between the overexpression micoarray results of LOXL2-delta72 and its wild type using the threshold of 1.5-fold change. We got 185 delta72-WT-DEGs, including 64 downregulated genes and 121 upregulated genes. These suggest that overexpression of LOXL2-delta72 caused the expression change of a great number of genes compared to its wild type, which provides important clues to reveal the potential functions

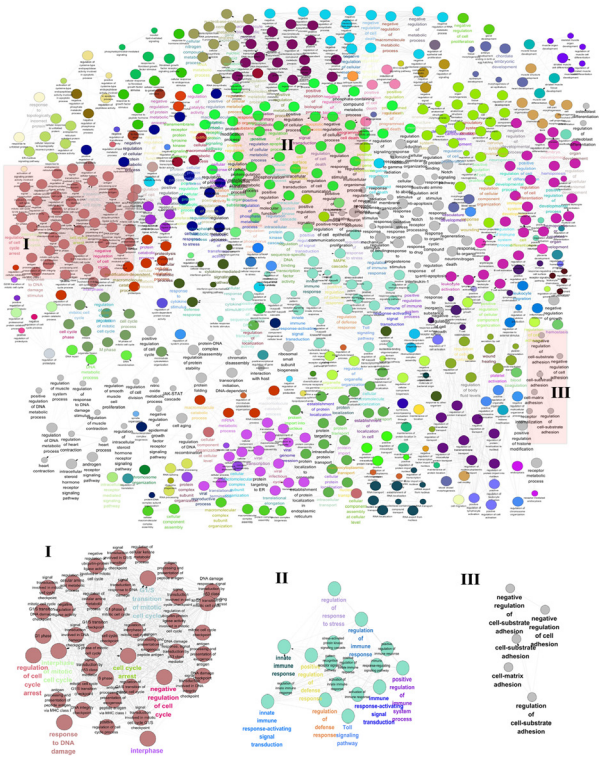

Figure 6. Functional Annotation Map of delta72-WT-DEGs PPI Network. The proteins in the delta72-WT-DEGs PPI network were represented as nodes corresponding to their associated enriched GO "Biological Process" terms, and the edges between GO terms indicate that some of their respective proteins share the same enriched GO terms. The significant "cell cycle", "immune" and "cell adhesion" related terms were indicated by light red shade and Roman number

and molecular mechanisms of LOXL2-delta72. To obtain a full view of influence of the delta72-WT-DEGs, its PPI sub-network was also constructed, which contained 2344 nodes and 42730 edges, including 100 upregulated genes and 54 downregulated genes (Figure 5A). Moreover, we found internal PPIs between the delta72-WT-DEGs, containing a small complex consisting of 17 DEGs and four pairs of protein-proteins interactions, such as upregulated PDIA3, INSIG2, ADM and OSMR, the downregulated TADA2A and RPS4X (Figure 5B). These suggested that certain proteins levels changed to favor the complex or interactions to achieve the influence of overexpression of LOXL2-delta72.

ClueGO creates a functionally organized GO category networks based on the overlaps between the different GO categories and the significance. To understand which aspects of cellular activities were affected by these DEGs through the interactions of PPI network, over-represented GO "Biological Process" terms of proteins in delta72-WT-DEGs PPI sub-network were analyzed. A functional annotation map containing 496 GO terms was generated by ClueGO, in which proteins of PPI sub-network were presented as nodes corresponding to their enriched GO terms, edges indicating that some of their respective proteins share the same enriched GO terms (Figure 6). A group of GO terms related to "cell cycle" were found, including "cell cycle arrest", "G1/S transition of mitotic cell cycle", "interphase" and "respond to DNA damage stimulus". Since LOXL2 is a secreted protein, its splicing variant LOXL2-delta72 also involved cell adhesion, such as "cell-matrix adhesion", "cell-substrate adhesion" and "negative regulation of cell adhesion". Surprisingly, this PPI sub-network was annotated by many significant "immunity" related terms, e.g. "innate immune response", "innate immune response-activating signal transduction", "regulation of defense response" and "Toll signaling pathway". These results suggested that the over-expression of LOXL2-delta72 affected specific various biological activities through the disturbed PPI sub-network, which provided a full view of its influence.

Discussion

Esophageal squamous cell carcinoma (ESCC), the most common histopathologic form of esophageal cancer, is one of the most prevalent cancers worldwide and is the fourth leading cause of cancer deaths in China (Ke et al., 2002; Parkin et al., 2005). Accumulated researches have shown that many genes generate alternative splicing in cancers (Venables, 2006; Skotheim et al., 2007). For example, breast cancer gene 1 (BRCA1) is responsible for the majority of hereditary breast and ovarian cancers. An inherited nonsense mutation in exon 18 of the BRCA1 gene disrupts an exonic splicing enhancer and leads to a variant of exon 18 skipping (Liu et al., 2001). On the other hand, RON receptor tyrosine kinase is a member of the MET proto-oncogene family that has been implicated in regulating motile-invasive phenotypes in certain types of epithelial cancers. Three splicing variants of RON (RONdelta165, RONdelta160, and RONdelta155) were generated by deletions in different regions in extracellular domains of the RON beta chain. Functional studies showed that expression of RONdelta160 or RONdelta155 in Martin-Darby canine kidney cells resulted in increased cell dissociation. RONdelta160 and RONdelta155 also exerted the ability to induce multiple focus formation and sustain anchorage-independent growth of transfected NIH3T3 cells (Zhou et al., 2003). These researches suggested the expression of alternative or even tumour-specific splice variants significantly affects many cellular events that are critical for cancer biology, such as cell proliferation, motility, and drug response.

Inspired by these evidences, LOXL2 splicing variant LOXL2-delta72 was overexpressed using plasmid followed by mRNA expression profile analysis to explore its biological roles. To gain a full view of the influences caused by the DEGs, their PPI sub-networks were generated by mapping them to public PPI dataset. The results showed that LOXL2WT-DEGs and LOXL2-delta72-DEGs interacted with thousands of other proteins, which suggested these DEGs have greatly impacted the cellular activities through their cascades of interactions. There were two evidences indicating that LOXL2-delta72 had different roles compared to its wild type. First, the comparison of two PPI sub-networks of LOXL2WT-DEGs and LOXL2-delta72-DEGs showed they contained hundreds of different proteins in the sub-network. Second, to illustrate the different roles of LOXL2-delta72 by the method of PPI sub-network, we selected the delta72-

WT-DEGs using the 1.5-fold change to generate the third PPI sub-networks, as the slight change of certain genes might also induce great change of cell molecular life. The delta72-WT-DEGs involved thousands of genes in its PPI sub-network, which suggests LOXL2-delta72 has a greatly different impact on ESCC cells compared to its wild type. Take the following several genes for example, PDIA3 (protein disulfide isomerase family A, member 3), upregulated 1.52-fold in this study, encodes a protein of the endoplasmic reticulum that interacts with lectin chaperones calreticulin and calnexin to modulate folding of newly synthesized glycoproteins. PDIA3 was significantly associated with clinicopathological features of both gallbladder squamous/adenosquamous carcinoma and gallbladder adenocarcinoma specimens, including high TNM stage and lymph node metastasis. PDIA3 was negatively correlated with poor postoperative patient survival and positively correlated with high mortality (Zou et al., 2013). INSIG2 (insulin induced gene 2) was overexpressed in colon cancer cells resulting in increased cellular proliferation, invasion, anchorage independent growth and inhibition of apoptosis. Over-expression of INSIG2 suppressed chemotherapeutic drug treatment-induced Bcl2 associated X protein (Bax) expression and activation (Li et al., 2008). Oncostatin M receptor (OSMR), with a fold change of 1.56 in this study, is commonly overexpressed in advanced cervical squamous cell carcinoma (SCC), producing a significantly worse clinical outcome. Overexpressed OSMR in cervical SCC cells activates TGM2/integrin-$\alpha 5\beta 1$ interactions and induces pro-malignant changes (Caffarel et al., 2013). These evidences indicated that the DEGs arised from the comparison of overexpression of LOXL2-delta72 and its wild type contains cancer critical genes which enable LOXL2-delta72 has distinguishing roles in ESCC.

The functional annotation of the PPI sub-network would provide important clues to explain the potential specific molecular mechanisms of LOXL2-delta72. So the ClueGO was applied to annotate the delta72-WT-DEGs PPI sub-network by over-represented GO "Biological Process" terms. The functional annotation map contained hundreds of significant GO terms, suggesting LOXL2-delta72 had a wide arrange of different impact compared to its wild type. Many GO terms related to cell cycle were found, including "cell cycle arrest", "G1/S transition of mitotic cell cycle", "interphase" and "respond to DNA damage stimulus". The PPI sub-network also involved cell adhesion, such as "cell-matrix adhesion", "cell-substrate adhesion" and "negative regulation of cell adhesion". One of the spotlights was the functional annotation map revealed many significant "immunity" related terms. The relationship between LOXL2 and immunity has not been reported before. However, our analyses results indicated that LOXL2-delta72 might impact the immune response of cancer cells in the tumor progression through its cascades of protein-protein interactions. These annotation results would guide us to test the biological roles of LOXL2-delta72 in the next stage.

In summary, we provided evidences to indicate that LOLX2-delta72 would play different roles compared to its wild type by the generation and analyses of PPI networks, which was more intuitive than the traditional list of their different genes. These results would be helpful in the experimental identification of its biological roles and explanation of molecular mechanisms. With the development of large-scaled and high-throughput techniques detecting protein-protein interacts, the public PPI network would become more reliable. The application of PPI network would be greatly expanded, such as transcriptome network analysis and pathway crosstalk analysis (Ma et al., 2012; Pan, 2012). Our analyses also provided a work flow to test the different roles of a splicing variant with high-throughput data.

Acknowledgements

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