Identification of Critical Genes and Proteins for Stent Restenosis Induced by Esophageal Benign Hyperplasia in Esophageal Cancer

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This study was conducted to explore the potential genes and proteins associated with esophagus benign hyperplasia induced by esophageal stents. Five patients with esophageal cancer subjected to esophageal stent placement were enrolled in this study. Long non-coding RNA (lncRNA) sequencing and tandem mass tag quantitative proteomics analysis were performed by using the collected hyperplastic samples and adjacent non-hyperplastic tissues. Differentially expressed (DE) RNAs and proteins were analyzed, followed by functional enrichment analysis, protein-protein interaction (PPI) network analysis, and competitive endogenous RNA (ceRNA) network construction. Venn analysis was performed to extract the overlaps between DE mRNAs and DE proteins and the expression correlations between DE mRNA and proteins were analyzed. Results showed that total 642 DE RNAs (457 mRNA and 185 lncRNAs) and 256 DE proteins were detected. DE mRNAs (such as MAOB, SDR16C5, and FOSL1) were enriched in oxidation-reduction process-associated functions. PPI network was comprised of 175 nodes and 425 edges. VEGFA was a significant node with the highest degree. LncRNA-mRNA network with three subnetworks (C1, C2, C3) was constructed for lncRNAs with more than 15 gene targets. RP11-58O9.2 was a significant lncRNA with the most target genes and RP11-667F14.1 regulated more than 20 targets. FOSL1 was a common target of the two lncRNAs. Function analysis showed that DE lncRNAs were involved in the HTLV-I infection (RP11-58O9.2 and RP11-667F14.1) and IL-17 signaling pathways (RP11-5Q24.1 and RP11-58O9.2). Total 11 DE mRNAs were overlapped with DE proteins, among which MAOB and SDR16C5 showed positive correlations between mRNA and protein expression. Function analysis showed that MAOB was enriched in oxidation-reduction process and its protein was closely related with response to lipopolysaccharide. VEGFA, FOSL1, MAOB, SDR16C5, RP11-58O9.2, RP11-667F14.1, and RP11-288A5.2 may be served as genetic targets for preventing stent restenosis in esophageal cancer.

Keywords: esophageal cancer, esophageal stents placement, esophagus restenosis, sequencing, proteomics
INTRODUCTION

Esophageal cancer is one of the most common human cancers globally and a leading cause of cancer-related deaths, with an estimated 400,000 deaths in 2012 (Ferlay et al., 2013). Esophageal cancer is typically asymptomatic and the disease has already progressed by the time the first symptoms appear. Therefore, more than half of the patients with esophageal cancer have advanced disease at the time of diagnosis (Pennathur et al., 2013). Dysphagia is the most common symptom of obstructive esophageal cancer (Mariette et al., 2007), which may lead to malnutrition and eventually result in a poor treatment response and poor prognosis (Mariette et al., 2012).

For patients with dysphagia, esophageal stent placement is a commonly used palliative treatment, which can quickly relieve the obstruction symptoms of patients, maintain oral intake, and reconstruct the gastrointestinal nutrition channel (van Heel et al., 2010a; Van Heel et al., 2010b). However, esophageal stent placement may result in recurrent dysphagia due to stent migration and tissue hyperplasia. Particularly, benign tissue hyperplasia-induced stent restenosis is the most intractable complication with an incidence of up to 46.1% (Hindy et al., 2012). Previous studies have reported that esophagus restenosis after esophageal stent placement is caused by fibroblast proliferation, which stimulates restenosis by delivering growth factors to monocytes (Marzocchi et al., 1991; Albiero et al., 2000; John et al., 2001). It has been reported that stents loaded with 125I seeds inhibit fibroblast proliferation compared to conventional stents (Gan et al., 2015). However, experiments using animal models indicated that 125I seeds cannot prevent benign tissue hyperplasia-induced stent restenosis (Guo et al., 2007). Therefore, investigating the mechanism of benign hyperplasia-induced stent restenosis is urgently needed to develop effective treatments.

In this study, we enrolled five patients with esophageal cancer who had undergone esphagus 125I stent placement and explored the potential genes and proteins associated with esophageus benign hyperplasia induced by esophageal stents. Transcriptome sequencing and tandem mass tag (TMT) quantitative proteomics analyses were performed for hyperplastic tissues and normal tissues of the esophageus wall. Transcriptome and proteomics data were analyzed by bioinformatics methods and validated by reverse transcription (RT)-PCR and western blot analyses.

RESULTS

Quality Control and Reference Genome Alignment

After removing low-quality reads, the clean reads were mapped to the human reference genome. The read mapping rate of samples ranged from 91.60 to 94.10%.

Differential Expression Analysis

Using thresholds of \(|\log_{2} FC| > 1\) and \(p\) value <0.05, 642 DE genes were identified, including 244 downregulated mRNAs, 213 upregulated mRNAs, 92 downregulated lncRNAs, and 93 upregulated lncRNAs. A heatmap of the DE genes is shown in Figure 1. All DE mRNAs and lncRNAs are shown in Supplementary Table 3.

Functional Enrichment Analysis of DE mRNAs

The DE mRNAs were enriched in 110 GO (BP, CC, and MF) terms or KEGG pathways, such as GO:0007155~cell adhesion, GO:0005576~extracellular region, GO:0055114~oxidation-reduction process, GO:0042542~response to hydrogen peroxide, and hsa05200:pathways in cancer. The top five terms ranked according to the increasing \(p\) values are shown in Figure 2.

PPI Network Construction

In the PPI network of mRNAs, there were 175 nodes and 435 edges. Network topological property analysis revealed that eight mRNAs [such as VEGFA, FOS, and MYC proto-oncogene, BHLH transcription factor (MTY)] had higher scores in three topological properties. The top 15 node genes with higher scores in the three topological properties are shown in Table 1.

Prediction of Target Genes of DE IncRNAs

A total of 151 DE lncRNAs were found to have target genes and lncRNAs that interacted with more than 15 genes were visualized in the lncRNA-mRNA network. As shown in Figure 3A, the network consisted of three parts (C1, C2, and C3), in which there were 1, 4, and 23 lncRNAs, respectively. The top ten lncRNAs that regulated the largest number of targets are shown in Table 2.

To understand the roles of DE lncRNAs, functional enrichment analyses were performed for their target genes. As shown in Figure 3B, the target genes were significantly enriched in the HTLV-I infection (RP11-5809.2 and RP11-667F14.1) and IL-17 signaling pathways (RP11-5O24.1 and RP11-5809.2). Additionally, both RP11-5809.2 and RP11-667F14.1 were associated with functions of response to glucocorticoid, and extracellular stimulus.

Function Similarity Analysis of IncRNAs

For the results obtained from GOSemSim, a Wang score >0.8 and Resnik score >0.5 were considered to have functional similarity between lncRNAs. The constructed functional similarity network showed that the upregulated CTB-131B5.2, RP11-635L1.2, and RP11-329B9.3 were linked with other lncRNAs in function (Figure 4).

miRNA Prediction and ceRNA Construction

Based on Figure 3A, the miRNAs were predicted for mRNAs in lncRNA-mRNA network. Results showed that seven miRNAs were predicted for mRNAs of C2 and three miRNAs were predicted for those of C3. No miRNAs were predicted for mRNAs in C1 network. Based on the miRanda database, nine miRNA-lncRNA interactions were predicted in C2 and 36 were predicted in C3. Finally, combining the miRNA-lncRNA, miRNA-mRNA and lncRNA-mRNA interactions, two ceRNA networks were
FIGURE 1 | Heatmaps of differentially expressed (A) lncRNAs and (B) mRNAs.

FIGURE 2 | The top 5 GO and KEGG pathway terms for differentially expressed mRNAs. The top five terms ranked according to the increasing p values. The black line indicates -log₁₀ (p value) for each term. BP, biological process; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes pathway; Count, number of genes enriched in one term.
TABLE 1 | Genes with higher degrees in the PPI network (top 15).

| Gene     | Degree | Gene     | Betweenness | Gene     | Closeness |
|----------|--------|----------|-------------|----------|-----------|
| VEGFA    | 39     | VEGFA    | 5672.6035   | VEGFA    | 0.0434674 |
| FOS      | 36     | FOS      | 4716.4443   | FOS      | 0.0433807 |
| MYC      | 35     | PTGS2    | 4104.0386   | JUN      | 0.04335908 |
| JUN      | 35     | MYC      | 3412.1555   | MYC      | 0.04330513 |
| PTGS2    | 30     | JUN      | 2487.5193   | PTGS2    | 0.04329435 |
| NGF      | 26     | NGF      | 2372.0730   | NGF      | 0.04313337 |
| EGR1     | 18     | BMP2     | 2067.0754   | SERPINE1 | 0.04284659 |
| ATF3     | 17     | MUC1     | 1897.5162   | MMP7     | 0.0428525 |
| SERPINE1 | 15     | MMP7     | 1769.2167   | DNAH8    | 0.04281496 |
| DUSP1    | 15     | GRIN2A   | 1556.0956   | EGR1     | 0.0427838 |
| CDKN1A   | 14     | DNAH8    | 1407.1766   | BMP2     | 0.04277286 |
| DNAH8    | 13     | GABRB2   | 1198        | ATF3     | 0.04273085 |
| AGTR1    | 13     | ALOX12B  | 1192        | DUSP1    | 0.04272036 |
| BMP2     | 12     | SLC2A1   | 963.84937   | PK3      | 0.04270987 |
| NR4A2    | 12     | AGTR1    | 945.83926   | HBEGF    | 0.04270987 |

constructed (Figure 5). The network of C2 consisted of 4 lncRNAs, 7 miRNAs, 22 mRNAs, and 115 interaction pairs. The C3 network included 21 lncRNAs, 3 miRNAs, 17 mRNAs, and 210 interaction pairs.

DE Protein Selection and Bioinformatics Analysis

For proteomics analysis, a total of 4546 proteins were obtained by searching in the UniProt database. After differential expression analysis, 256 DE proteins were identified (Supplementary Table 3).

PPI network was constructed with protein pairs (Figure 6A). In this network, acetyl-CoA carboxylase alpha (ACACA), enolase 2 (ENO2), phosphoribosylformylglycinamidine synthase (PFAS), glutamyl-prolyl-tRNA synthetase (EPRS), spectrin alpha, non-erythrocytic 1 (SPTAN1), RNA polymerase II subunit C (POLR2C), RNA binding motif protein 8A (RBM8A), Erb-B2 receptor tyrosine kinase 2 (ERBB2), S100 calcium binding protein B (S100B), and dicer 1, ribonuclease III (DICER1) were in the top 15 genes for three topological properties.

GO analysis revealed that the DE proteins were closely related to oxidation-reduction process, protein binding, and extracellular exosome (Figure 6B). Moreover, the DE proteins were significantly involved in hsa01100:metabolic pathways, and hsa01130:biosynthesis of antibiotics (Figure 6C).

Conjoint Analysis of Proteome and Transcriptome Data

The DE genes and DE proteins with p < 0.05 identified above were subjected to Pearson correlation analysis. The expression correlation analysis showed that the R values of most genes and proteins ranged from 0.7 to 1.0, suggesting that most genes and proteins had positive correlation in expression level. In addition, analysis of the correlation of the fold-change between DE mRNAs and DE proteins also showed positive correlation with R = 0.57 and p value <2.2e-16 (Figure 7), suggesting the consistency of gene and protein expression. Furthermore, Venn analysis identified 11 overlapped proteins/miRNAs [MAOB, Fc fragment of IgG binding protein (FCGBP), OCA domain containing 2 (OCIAD2), collagen type I alpha 1 chain (COL1A1), COL7A1, glutamine-fructose-6-phosphate transaminase 1 (GPT1), asparaginase (ASPG), proliferation and apoptosis adaptor protein 15 (PEA15), SDR16C5, secernin 1 (SCRN1), and microfibril associated protein 2 (MFAP2)]. Correlation analysis showed that there were positive correlations between protein and mRNA for FCGBP, MAOB and PEA15 (Table 3).

RT-PCR Validation

The expression levels of RP11-58O9.2, RP11-667F14.1, VEGFA, and FOSL1 in hyperplastic tissues were significantly higher than in normal tissues, which was consistent with the differential protein analysis above (P < 0.05). Additionally, compared to normal tissues, RP11-288A5.2, MAOB, and SDR16C5 were significantly downregulated in hyperplastic tissues, which were in accordance with the prediction analysis (P < 0.05) (Figure 8A). However, CTD-2350J17.1 was not detected in hyperplastic tissues.

Western Blotting Validation

The protein expression levels of MAOB and SDR16C5 are shown in Figure 8B. Compared with normal tissues, the two proteins above were downregulated in hyperplastic tissues.

DISCUSSION

Currently, esophageal stents have been increasingly used for palliation of malignant dysphagia and are the most common means of palliation (Sharma et al., 2002; Sharma and Kozarek, 2010). Nevertheless, tissue benign hyperplasia-induced stent restenosis is a common complication influencing the long-term effect of stent placement, however, no methods have been developed to overcome this problem. In this study,
FIGURE 3 | (A) LncRNA regulatory network; (B) Pathways enriched by lncRNAs. Red triangle, upregulated lncRNA; red circle, upregulated mRNAs; green triangle, downregulated lncRNAs; green circle, downregulated mRNAs.
TABLE 2 | The top ten lncRNAs that regulated more targets.

| lncRNA         | Freq |
|----------------|------|
| RP11-58O9.2    | 36   |
| CTD-3247F14.2  | 33   |
| RP11-63S1L.2   | 33   |
| RP11-1100L3.8  | 32   |
| RP11-63SN19.3  | 31   |
| RP11-61J19.5   | 27   |
| RP11-667F14.1  | 27   |
| CTB-131B5.2    | 26   |
| LINC01220      | 26   |
| MIF24-2        | 26   |

we combined high-throughput sequencing and proteomics technologies to explore the genetic and protein markers associated with benign hyperplasia caused by restenosis after esophageal stent placement.

A previous study reported that restenosis after percutaneous intervention is characterized by growth factor release, platelet aggregation, inflammatory cell infiltration, extracellular matrix remodeling, medial, and smooth muscle and endothelial cell proliferation and migration (Marx et al., 2011). VEGF is a growth factor that induces the proliferation and migration of vascular endothelial cells (Leung et al., 1990). Additionally, Luttun et al. (2002) reported that VEGF may promote the development of in-stent restenosis via proinflammatory effects. A recent study showed that VEGF was upregulated in a group of patients who developed in-stent restenosis (Katsaros et al., 2013). In our study, VEGFA, a member of the VEGF growth factor family, was upregulated in hyperplasia tissues compared to normal tissues. Shu et al. (2014) suggested that VEGFA stimulates endothelial cell proliferation. Furthermore, higher VEGF levels after percutaneous coronary intervention was reported to be associated with restenosis (Katsaros et al., 2013). Therefore, VEGFA may be a key marker of benign hyperplasia-induced restenosis.

In this study, MAOB, SDR16C5 and FOSL1 were found to be involved in oxidation-reduction associated functions, such as GO:0055114~oxidation-reduction process (MAOB, and SDR16C5), GO:0016491~oxidoreductase activity (MAOB), and GO:0042542~response to hydrogen peroxide (FOSL1). Specifically, MAOB and SDR16C5 were predicted in both the transcriptome and proteomics data and presented positive correlations in mRNA and protein expression. It has been suggested that restenosis is a variable combination of de novo proliferative and remodeling processes with neoplastic features, as well as homeostatic repair of the vessel wall (Libby and Tanaka, 1997). Therefore, vessel injury repair may be a key determinant of restenosis. Stone and Collins (2002) have reported that a low concentration of hydrogen peroxide stimulates the proliferation and migration of endothelial cells. Additionally, oxidation-reduction process plays a prominent role in tissue injury and vascular cell signaling, which is involved repairing vessel injury (Azevedo et al., 2000). Therefore, MAOB, SDR16C5, and FOSL1 may play key roles in esophageal restenosis involving oxidation-reduction processes.

In the constructed lncRNA-mRNA network, RP11-58O9.2 regulated the largest number of target genes. Additionally, RP11-667F14.1 regulated more than 20 targets. Interestingly, FOSL1 was a common target of the two lncRNAs, suggesting that RP11-58O9.2 and RP11-667F14.1 play significant roles in hyperplastic tissue development by regulating FOSL1. Functional analysis showed that RP11-58O9.2 is involved in the IL-17 signaling pathway. Interleukin (IL)-17 may stimulate several types of cells to secrete multiple proinflammatory mediators. Local production of IL-17 may cause site-specific influx and activation of inflammatory cells (Fossiez et al., 1998). Importantly, studies have supported a key role for inflammatory cells in the restenosis process (Welt and Rogers, 2002). Additionally, RP11-58O9.2 and RP11-667F14.1 were significantly enriched in the pathway of HTLV-1 infection. A previous study has investigated the correlation between HTLV-1 and esophageal squamous cell carcinoma (Mirsadraee et al., 2007), but no correlation is found. In this study, we did not test HTLV-1 in patients. Thus, the role of HTLV-1 infection in esophageal cancer needed to be further investigated.

Furthermore, both RP11-288A5.2 and CTD-2350J17.1 regulated MAOB with the top two lower adjusted p values. Specifically, the expression of RP11-288A5.2 was verified. As described above, MAOB was enriched in functions associated with oxidation-reduction process. Additionally, this protein was involved in the GO:0032496~response to lipopolysaccharide (LPS). LPS is a large molecule consisting of a lipid and polysaccharide, which acts as an endotoxin to elicit strong immune responses in animals. Moreover, LPS induces extracellular matrix degradation and stimulates the production of various cytokines (Aota et al., 2006). Forrester et al. (1991) suggested that there were three phases in the restenosis process, including an inflammatory phase, cellular proliferation phase, and remodeling of extracellular matrix protein synthesis phase, which indicates an important role for extracellular matrix remodeling in restenosis. Additionally, the extracellular matrix is the basis for lesion growth. The aortic hyperplasia is related to extracellular matrix deposition (Dao et al., 2001). Therefore, RP11-288A5.2 and CTD-2350J17.1 may be associated with restenosis by participating in extracellular matrix remodeling. The expression and role of CTD-2350J17.1 in benign hyperplasia-induced restenosis requires further analysis.

Besides, the pathway analysis showed that DE proteins in hyperplasia tissues compared with normal tissues in patients with esophageal cancer were significantly enriched in the biosynthesis of antibiotics pathway. A previous report suggested that the biosynthesis of antibiotics and other secondary metabolites was controlled by inorganic phosphate (Martin, 2004). The alterations in carbohydrate metabolism are present in endometrial hyperplasia and endometrial carcinoma patients (Benjamin and Casper, 1966). The level of inorganic phosphate in blood plasma is related with glucose metabolism underlying
glucose use. The level of phosphate has been considered as an index of peripheral use of glucose. In addition, the global gene analysis indicated the antibiotic biosynthetic pathways in *Streptomyces* (Huang et al., 2001). In the present study, after sequencing, the data of each sample were subjected to comparative analysis and the results showed that all the data were reliable, which suggested that there was no bacterial contamination in the process of experiments. Thus, we suggested that the dysregulation of biosynthesis of antibiotics pathway may be related with the glucose metabolism in the hyperplasia tissues.

In conclusion, we combined transcriptome and proteomics data to investigate the critical genes/proteins associated with benign hyperplasia-induced restenosis in patients with esophageal cancer. DE VEGFA, FOSL1, MAOB, SDR16C5, RP11-58O9.2, RP11-667F14.1, and RP11-288A5.2 in hyperplastic tissues may serve as genetic targets for preventing stent restenosis in esophageal cancer. However, the sample size used in this study was a little small, so we will collect more samples to confirm our results in the future study.

**MATERIALS AND METHODS**

**Patients and Samples**

From April to July in 2017, five patients (Supplementary Table 1) with esophageal cancer admitted to our hospital were enrolled in the study. These patients were subjected to $^{125}$I particle esophagus stent placement for palliation of dysphagia due to esophageal cancer and presented with tissue hyperplasia around the perforation of the stent several months after stent placement. Five pairs of hyperplastic tissues and adjacent non-hyperplastic tissues (normal tissues) were collected during surgery, among which four pairs were used for lncRNA sequencing and all five pairs were used for proteomics. Our study was approved by the
ethics committee of our hospital. All patients provided informed consent before the experiments.

**Total RNA Extraction and RNA Sequencing**

Total RNA was extracted from four pairs of hyperplastic tissues and normal tissues using TRIzol reagent (Takara, Shiga, Japan) according to the manufacturer's instructions. The quality and concentration of RNA samples were, respectively, determined with a NanoDrop spectrophotometer (Wilmington, DE, United States) and Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, United States). The quality of RNA samples is shown in Supplementary Table 2. RNA integrity was detected with an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, United States). Ribosomal RNA was removed from total RNA samples by using a Ribo-zero rRNA Removal Kit (EPICENTRE, Madison, WI, United States). Strand-specific libraries were constructed by the dUTP method using the NEB Next Ultra Directional RNA Library Prep Kit for Illumina (NEB, Ipswich, MA, United States). The libraries were sequenced on an Illumina HiSeq 2500 platform with 150-base pair single-end reads.

**Quality Control and Reference Genome Alignment**

Trimmomatic (version 3.6) (Bolger et al., 2014) tool was used for quality control of raw reads. Unreliable bases and low-quality reads were removed to obtain clean reads. The clean reads were mapped to the human genome (GRCh38, p7, GENCODE) (Harrow et al., 2012) using TopHat2 (version 2.1.0) (Trapnell et al., 2009) and the lncRNA and mRNA information was obtained.

The sequencing data are deposited in the NCBI Sequence Read Archive (SRA) database with the accession number PRJNA544132.

**Analysis of Gene Expression Levels and Differentially Expressed (DE) Genes**

The gene expression level was determined by estimating the reads located in genomic regions or gene exon regions. Based on the human genome annotation information in the GENCODE database, the count of reads mapped to the genes was obtained using HTSeq (version 0.6.1p2) (Anders et al., 2015), which was then normalized using the counts per million method. Additionally, genes with a CMP value of <0.1 in at least three samples were defined as low expression abundance genes. The obtained genes were divided into lncRNA and mRNA according to the gene type in the annotation information.

Differential expression analysis for lncRNAs and mRNAs was performed using likelihood ratio tests in edgeR of R (Lun et al., 2016). DE lncRNAs and mRNAs between paired hyperplastic tissues and normal tissues were analyzed. Moreover, RNAs with low expression abundance were deleted before DE analysis. A |logFC (fold change)| > 1 and p value < 0.05 were used as thresholds of DE analysis.

**Functional Enrichment Analysis of DE mRNAs**

The DE mRNAs were subjected to Gene Ontology (GO) function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses by using the DAVID (version 6.8) online tool (Huang et al., 2008). The significantly enriched GO terms and pathways were selected with the thresholds of a count ≥2 and p value <0.05.

**Protein-Protein Interaction (PPI) Network Analysis**

The PPIs of DE mRNAs were analyzed using the online tool STRING (Szklarczyk et al., 2015). Required confidence (combined score) > 0.4 was selected as the threshold of PPI. Next, the network based on the PPI was constructed using
LncRNA-mRNA Interaction Network Analysis

The correlations between the obtained DE lncRNAs and mRNAs were analyzed by calculating the Pearson correlation coefficients (Pearson, 2006). The p values were adjusted using the Benjamini and Hochberg (1995) method. An adjusted p value < 0.01 indicated a correlation between DE lncRNAs and mRNAs and the mRNAs were considered as potential target genes of lncRNAs. Finally, the lncRNA-target gene regulatory network was constructed using Cytoscape software (Shannon et al., 2003).

Function Analysis of lncRNAs

The functions of DE lncRNAs were predicted through GO and KEGG analyses of their target genes. In this study, lncRNAs with ≥ 15 targets were subjected to cellular component (CC), molecular function (MF), biological process (BP), and pathway prediction using clusterProfiler (Yu et al., 2012) with an adjusted p value < 0.05.

The GO function (BP) similarity between lncRNA was determined using Resnik method [Resnik method (Poesio, 1999) and Wang method (Wang et al., 2007)] provided by GOSemSim (Yu et al., 2010). Resnik method is an information content-based method depending on the frequencies of the two GO terms involved and that of their closest common ancestor term in a specific corpus of GO annotations; the Wang method is a graph-based method that uses the topology of the GO graph structure to compute semantic similarity.

miRNAs Prediction and Competitive Endogenous RNA (ceRNA) Network Construction

Based on the lncRNA-mRNA interactions, we predicted the miRNAs that regulated the mRNAs using TargetScan_microrNA_2017 in Enrichr (Chen et al., 2013). The miRNA-mRNA regulatory pair with a p value < 0.01 was selected. Next, the interactions between the predicted miRNAs and obtained lncRNAs were predicted using miRanda (Enright et al., 2003). If there was a binding site between an miRNA and obtained lncRNA, we considered that there was a regulatory interaction between these factors. Based on the miRNA-lncRNA, miRNA-mRNA, and lncRNA-mRNA interactions, a ceRNA network was constructed using Cytoscape software.

TMT Quantitative Proteomics

Proteins between five pairs of hyperplastic tissues and normal tissues were analyzed by tandem mass tag technology. Briefly, protein was extracted from five pairs of tissues using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) and quantified using the BCA® Protein Assay Kit (Pierce, Madison, WI, United States). The quality of protein samples was evaluated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The qualified proteins were subjected to reductive alkylation. Then trypsin was added at an enzyme to protein ratio of 1:50 (w/w) and incubated at 37°C overnight. Following that the peptides were dissolved in 0.5 M tetraethyl-ammonium bromide and then labeled with TMT. The five samples in

![FIGURE 6](image_url)

Cytoscape (Shannon et al., 2003), and topological properties [degree centrality (Opsahl et al., 2010), betweenness centrality (Cukierski and Foran, 2008), and closeness centrality (Du et al., 2015)] of the network were analyzed using CytoNCA (Tang et al., 2015) in Cytoscape to identify hub nodes in the PPI network.
FIGURE 7 | Correlation between the mRNA and protein expression levels.

TABLE 3 | Correlation analysis results of overlapped genes/proteins.

| Uniprot_id | Gene_names | Rho     | Description                                                                 |
|------------|------------|---------|-----------------------------------------------------------------------------|
| A0A087WXI2 | FCGBP      | 0.838689066 | IgGFc-binding protein OS = Homo sapiens GN = FCGBP PE = 1 SV = 1            |
| P27338     | MAOB       | 0.78397768  | Amine oxidase [flavin-containing] B OS = Homo sapiens GN = MAOB PE = 1 SV = 3 |
| Q15121     | PEA15      | 0.828973806 | Astrocytic phosphoprotein PEA-15 OS = Homo sapiens GN = PEA15 PE = 1 SV = 2   |

Protein Identification and Quantification

LC-MS/MS data were analyzed using Proteome Discoverer™ Software 2.1 interfaced with UniProt uniprot-proteome-UP000005640-Homosapiens database (The UniProt Consortium, 2017) at 20170912. In order to avoid precursor ion interference, the trypsin was added at an enzyme to protein ratio of 1:50 (w/w) and incubated at 37°C overnight. The polypeptide samples were re-dissolved in UPLC loading buffer and separated by high-performance liquid chromatography with C18 reverse phase column. Total 71591 human protein sequences in FASTA format were searched. The searched parameters included two maximal missed cleavages, fixed modifications [Carbamidomethyl (C), TMT 6plex(K), TMT 6plex (N-Terminus)], variable modifications [Oxidation(M), Acetyl (Protein N-Terminus)], Mass tolerance for precursor ions (20 ppm), Mass tolerance for fragment ions (0.02 Da). Percolator node within Proteome Discoverer were used to calculate the false discovery rates (FDRs) for Peptide-spectrum matches (PSMs). PSMs were filtered by 7 minimal peptide length, mass accuracy (~2.5 ppm) and matching scores to achieve 1% protein FDR.

All the peptide sequences and proteins identification were listed in the Supplementary Files 1, 2. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2018) partner repository with the dataset identifier PXD014242.

DE Protein Identification and Bioinformatics Analysis

The quantitative data of protein abundance in the two groups of matched samples were normalized with loess method.
FIGURE 8 | (A) Relative expression of VEGFA, FOSL1, MAOB, SDR16C5, RP11-5809.2, RP11-667F14.1, and RP11-288A5.2 detected by RT-PCR analysis; (B) Protein levels of MAOB and SDR16C5 detected by western blot. *p < 0.05, compared to normal tissues; **p < 0.01, compared to normal tissues.

TABLE 4 | Primer sequence for RT-PCR.

| Primer   | Sequences (5′-3′)                      |
|----------|----------------------------------------|
| GAPDH-hF | TGACACCTTTGATACGTGAAAGGG               |
| GAPDH-hR | AGCCAGGGTGTGTTCTGAGGAGG                |
| RP11-288A5.2-hF | CCGCTCTTCCTCCTCCATA                   |
| RP11-288A5.2-hR | CCTAACGAGGCGCCTTC                |
| CTD-2350J17.1-hF | CCCTTATTCTGCTCTC                  |
| CTD-2350J17.1-hR | CAGAAGTTGAGTTGCTCTG               |
| RP11-5809.2-hF | TGCGCTGACCTATGTGAC              |
| RP11-5809.2-hR | AGGTTCCGCTAAACCTTC               |
| RP11-667F14.1-hF | AGCGAAGTGACGGGTCCTCG              |
| RP11-667F14.1-hR | ATCCGCTGATGATCTGTTT             |
| VEGFA-hF | CCGCTCGCTCTGCTAC                   |
| VEGFA-hR | CTCGATTGATGCGAGATGC              |
| FOSL1-hF | TCCCTGGGGTATTGGGAGAT             |
| FOSL1-hR | CCTACGAGGCTCCTGAGG            |
| MAOB-hF | GTGCGGATACGCTCGAAGC          |
| MAOB-hR | GGTCTCCAATCCTAGGATG         |
| SDR16C5-hF | TATACCTGCGATTTGGAAGCG     |
| SDR16C5-hR | CGATTCCGATGTCGTTTGTGAAGG    |

DE proteins were then subjected to PPI analysis using STRING, as well as GO and KEGG pathway analyses with DAVID 6.8 as described above.

Conjoint Analysis of Proteome and Transcriptome Data

Based on the obtained proteome and transcriptome data, we selected the common proteins/genes and calculated the correlation between the proteins and genes based on expression levels using the Pearson correlation coefficient. Results with p < 0.05 were considered reliable. Additionally, we performed VENN analysis for the DE proteins and mRNAs to screen for overlapped DE proteins/mRNAs. The Pearson correlation between the overlapped DE proteins and mRNAs in expression abundance was also analyzed.

RT-PCR Validation

Total RNA was isolated from hyperplastic tissues and normal tissues (50–100 mg) using the TRIzol method. After quality and purity evaluation, the reverse transcription reaction was performed with 0.5 μg RNA by using PrimeScript RT Master Mix (RR036A, Takara). Real-time PCR analysis was performed in a reaction volume of 20 μL (10 μL SYBR Premix EX Taq (2×), 1 μL forward primer, 1 μL reverse primer, and 8 μL cDNA). PCR amplification was performed as follows: 50°C for 3 min, 95°C for 3 min, and 40 cycles of 95°C for 10 s and 60°C for
30 s. The expression levels of RP11-288A5.2, RP11-58O9.2, RP11-667F14.1, CTD-2350J17.1, monoamine oxidase B (MAOB), short chain dehydrogenase/reductase family 16C member 5 (SDR16C5), vascular endothelial growth factor A (VEGFA), and FOS like 1, AP-1 transcription factor subunit (FOSL1) were detected. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primer sequences are listed in Table 4.

**Western Blotting Validation**

Proteins were extracted from hyperplastic tissues and normal tissues with RIPA buffer (P0013B; Beyotime, Shanghai, China). Protein concentrations were measured with BCA method using a BCA protein assay kit (PL212989; Thermo, United States). After denaturation, protein samples were subjected to SDS-PAGE and blotted onto polyvinylidene fluoride membranes (IPVH00010; Millipore, United States). Non-specific binding sites were blocked with 5% skim milk. Following washing with 1 × PBS-T (1000 mL 1 × PBS + 1 mL Tween-20) for three times, the membranes were incubated with primary antibodies [1:1000; MAOB (ab88510; Abcam, United States) and SDR16C5 (Cat #PA5-31421; Thermo)], or β-actin (Sc-47778; Santa Cruz Biotechnology, United States) overnight at 4°C, followed by washing six times in 1 × PBS-T. Then the membranes were incubated with horseradish peroxidase conjugated secondary antibodies [anti-mouse (1:5000) or anti-rabbit IgG (1:10000)] for 2 h at 37°C and washed for six times with 1 × PBS-T. Finally, the membranes were visualized by ECL detection (Millipore, United States).

**Statistical Analysis**

The experiments of RT-PCR and western blotting were repeated three times. Data were shown as mean ± standard deviation (SD). GraphPad Prism 5 (San Diego, CA) was used to analyze the data from this study. Student's t-test was used for comparison. Statistical significance was considered for p-value >0.05.

**DATA AVAILABILITY STATEMENT**

The sequencing data have been deposited in the NCBI Sequence Read Archive (SRA) database with the accession number PRJNA544132. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD014242.

**ETHICS STATEMENT**

The studies involving human participants were reviewed and approved by Ethics Committee of Tongren Hospital, Shanghai Jiao Tong University School of Medicine. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

**AUTHOR CONTRIBUTIONS**

AM and XZ: conception and design of the research, obtaining funding, and revision of manuscript for important intellectual content. MS, SW, and XY: acquisition of data. MS, BL, and XY: analysis and interpretation of data. MS and XY: statistical analysis. LW and SS: drafting the manuscript. All authors read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.563954/full#supplementary-material

**REFERENCES**

Albiero, R., Adamian, M., Kobayashi, N., Amato, A., Vaghetto, M., Di, M. C., et al. (2000). Short- and intermediate-term results of (32)P radioactive beta-emitting stent implantation in patients with coronary artery disease: the Milan Dose-response study. *Circulation* 101, 18–26. doi: 10.1161/01.cir.101.1.118

Anders, S., Pyl, P. T., and Huber, W. (2015). HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics* 31, 166–169. doi: 10.1093/bioinformatics/btu638

Aota, Y., An, H. S., Imai, Y., Thonar, E. J., Muehleman, C., and Masuda, K. (2006). Comparison of cellular response in bovine intervertebral disc cells and articular chondrocytes: effects of lipopolysaccharide on proteoglycan metabolism. *Cell Tissue Res.* 326, 787–793. doi: 10.1007/s00441-006-0225-1

Azevedo, L. C. P., Pedro, M. D. A., Souza, L. C., de Souza, H. P., Janiszewski, M., da Luz, P. L., et al. (2000). Oxidative stress as a signaling mechanism of the vascular response to injuryThe redox hypothesis of restenosis. *Cardiovasc. Res.* 47, 436–445. doi: 10.1016/s0008-6363(00)0091-2

Benjamin, F., and Casper, D. J. (1966). Alterations in carbohydrate metabolism induced by progesterone in cases of endometrial carcinoma and hyperplasia. *Am. J. Obstetr. Gynecol.* 94, 991–996. doi: 10.1016/0002-9378(66)9038-x
Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. 57, 289–300. doi:10.1111/j.2517-6161.1995.tb02031.x

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Chen, E. Y., Tan, C. M., Yan, K., Duan, Q., Wang, Z., Meirelles, G. V., et al. (2013). Enrichr: interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinform. 14:128. doi: 10.1186/1471-2105-14-128

Cleveland, W., Grosse, E., and Shyu, W. (1992). “Using betweeness centrality to identify manifold shortcuts,” in Proceedings of the IEEE International Conference on Data Mining Workshops. (Pisa: IEEE), 949–958.

Dao, H. H., Lemay, J., DeC, C. J., Debois, D., and Moreau, P. (2001). Norepinephrine-induced aortic hyperplasia and extracellular matrix deposition are endothelin-dependent. J. Hypertens. 19, 1965–1973. doi: 10.1097/00004872-200111000-00006

Duan, Y., Gao, C., Chen, X., Hu, Y., Sadiq, R., and Deng, Y. (2015). A new closeness centrality measure via effective distance in complex networks. Chaos 25, 440–442.

Enright, A. J., John, B., Gaul, U., Tuschl, T., Sander, C., and Marks, D. S. (2003). MicroRNA targets in Drosophila. Genome Biol. 5, 2003–2005.

Forrester, J. S., Fishbein, M., Helfant, R., and Fagin, J. (1991). A paradigm for restenosis based on cell biology: clues for the development of new preventive therapies. J. Am. College Cardiol. 17, 758–769. doi:10.1016/0735-1097(91)00196-2

Fossiez, F., Banchereau, J., Murray, R., Van, K. C., Garrone, P., and Lebecque, S. (1998). Interleukin-17. Int. Rev. Immunol. 16, 541–551.

Gar, Z., Jing, J., Zhu, G., Qin, Y., Teng, G., and Guo, J. (2015). Preventive effects of 125I seeds in a dog model. Mol. Med. Rep. 11, 3382–3390. doi:10.3892/mmr.2014.4310

Guo, J. H., Teng, G. J., Zhu, G. Y., He, S. C., Deng, G., and He, J. (2007). Self-expandable stent loaded with 125I seeds: feasibility and safety in a rabbit model. Eur. J. Radiol. 61, 356–361. doi:10.1016/j.ejrad.2006.10.003

Harrow, J., Frankish, A., Gonzalez, J. M., Tapanari, E., Diekhans, M., Kokocinski, F., et al. (2012). GENCODE: the reference human genome annotation for The ENCODE Project. Genome Res. 22, 1760–1774. doi:10.1101/gr.135390.111

Hindy, P., Hong, J., Lam-Tsai, Y., and Gress, F. (2012). A comprehensive review of esophageal stents. Gastroenterol. Hepatol. 8, 526.

Huang, D. W., Sherman, B. T., and Lempicki, R. A. (2008). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4:44. doi:10.1038/nprot.2008.211

Huang, J., Lih, C.-J., Pan, K.-H., and Cheng, C. S.-N. (2001). Global analysis of growth proliferative responses. J. Hypertens. 19, 758–769. doi:10.1016/s0360-3016(01)01822-3

Katsaros, K. M., Kastl, S. P., Krychtiuk, K. A., Hutter, R., Zorn, G., Maurer, G., et al. (2013). An increase of VEGF plasma levels is associated with restenosis of coronary model. Circ. Cardiovasc. Interv. 6, 104–111.

Mariette, C., De Botton, M. L., and Piessen, G. (2012). Surgery in esophageal and gastric cancer patients: what is the role for nutrition support in your daily practice? Ann. Surg. Oncol. 19, 2128–2134. doi:10.1245/s10434-012-2225-6

Martin, J. F. (2004). Phosphate control of the biosynthesis of antibiotics and other secondary metabolites is mediated by the PhoR-PhoP system: an unfinished story. J. bacteriol. 186, 5197–5201. doi:10.1128/jb.186.11.5197-5201.2004

Marx, S. O., Totary-Jain, H., and Marks, A. R. (2011). Vascular smooth muscle cell proliferation in restenosis. Circ. Cardiovasc. Interv. 4, 104–111.

Sharma, V., Mahantshetty, U., Dinshaw, K. A., Deshpande, R., and Sharma, S. (2010). Role of esophageal stents in benign and malignant diseases. Am. J. Gastroenterol. 105, 258–273. doi:10.1038/ajg.2009.684

Sharma, V., Mahantshetty, U., Dinshaw, K. A., Deshpande, R., and Sharma, S. (2002). Palliation of advanced/recurrent esophageal carcinoma with high-dose-rate brachytherapy. Int. J. Radiat. Oncol. Biol. Phys. 52, 310–315. doi: 10.1016/s0360-3016(01)01822-3

Sharma, P., Kozarek, R. (2010). Role of esophageal stents in benign and malignant diseases. Am. J. Gastroenterol. 105, 258–273. doi:10.1038/ajg.2009.684

Sharma, V., Khalsa, K. A., Deshpande, R., and Sharma, S. (2002). Palliation of advanced/recurrent esophageal carcinoma with high-dose-rate brachytherapy. Int. J. Radiat. Oncol. Biol. Phys. 52, 310–315. doi:10.1016/s0360-3016(01)01822-3
The UniProt Consortium (2017). UniProt: the universal protein knowledgebase. Nucleic Acids Res. 45, D158–D169. doi: 10.1093/nar/gkw1099
Trapnell, C., Pachter, L., and Salzberg, S. L. (2009). TopHat: discovering splice junctions with RNA-Seq. Bioinformatics 25, 1105–1111. doi: 10.1093/bioinformatics/btp120
van Heel, N. C., Haringsma, J., Spaander, M. C., Bruno, M. J., and Kuipers, E. J. (2010a). Esophageal stents for the relief of malignant dysphagia due to extrinsic compression. Endoscopy 42:536. doi: 10.1055/s-0029-1244123
Van Heel, N. C., Haringsma, J., Spaander, M. C., Didden, P., Bruno, M. J., and Kuipers, E. J. (2010b). Esophageal stents for the palliation of malignant dysphagia and fistula recurrence after esophagectomy. Gastrointest. Endosc. 72, 249–254. doi: 10.1016/j.gie.2010.01.070
Wang, J. Z., Du, Z., Payattakool, R., Yu, P. S., and Chen, C. F. (2007). A new method to measure the semantic similarity of GO terms. Bioinformatics 23, 1274–1281. doi: 10.1093/bioinformatics/btm087
Welt, F. G. P., and Rogers, C. (2002). Inflammation and restenosis in the stent era. Arterioscler. Thromb. Vasc. Biol. 22, 1769–1776. doi: 10.1161/01.atv.0000037100.44766.5b
Yu, G., Li, F., Qin, Y., Bo, X., Wu, Y., and Wang, S. (2010). GOSeSim: an R package for measuring semantic similarity among GO terms and gene products. Bioinformatics 26, 976–978. doi: 10.1093/bioinformatics/btp084
Yu, G., Wang, L. G., Han, Y., and He, Q. Y. (2012). clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS 16, 284–287. doi: 10.1089/omi.2011.0118
Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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