Alteration in gene expression profile and oncogenicity of esophageal squamous cell carcinoma by RIZ1 upregulation

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Tumor development was quantified, and changes in gene expression of RIZ1 transfected tumors were detected by RT-PCR and Western blotting.

RESULTS: DNA microarray data showed that RIZ1 transfection induced widespread changes in gene expression profile of cell line TE13, with 960 genes upregulated and 1163 downregulated. Treatment of tumor xenografts with RIZ1 recombinant plasmid significantly inhibited tumor growth, decreased tumor size, and increased expression of RIZ1 mRNA compared to control groups. The changes in gene expression profile were also observed in vivo after RIZ1 transfection. Most of the differentially expressed genes were associated with cell development, supervision of viral replication, lymphocyte costimulatory, and immune system development in esophageal cells. RIZ1 gene may be involved in multiple cancer pathways, such as cytokine receptor interaction and transforming growth factor beta signaling.

CONCLUSION: The development and progression of esophageal cancer are related to the inactivation of RIZ1. Virus infection may also be an important factor.

Key words: Retinoblastoma protein-interacting zinc finger gene 1; Microarray; Nude mice; Esophageal squamous cell carcinoma cells

Core tip: Retinoblastoma protein-interacting zinc finger gene 1 (RIZ1) transfection induced widespread changes in gene expression profile of cell line TE13, with 960 genes upregulated and 1163 downregulated. Most of the differentially expressed genes are associated with cell development, supervision of viral replication, lymphocyte costimulatory, and immune system development in esophageal cells. RIZ1 gene may be involved in multiple cancer pathways, such as cytokine receptor interaction and transforming growth factor beta signaling. Virus infection may also be an important factor in
Introduction

Esophageal cancer is one of the most common forms of cancer in the world and is a leading cause of cancer deaths in China and other developing countries. To date, the mechanisms of esophageal cancer are unclear. Tumor occurrence and development are regulated by a variety of oncogenes and tumor suppressor genes,[13] including the putative tumor suppressor gene, Retinoblastoma protein-interacting zinc finger gene 1 (RIZ1). The RIZ gene has two expression products: RIZ1, which is believed to be a histone methyltransferase and acts on the locus of H3K9; and RIZ2, which lacks the PR-domain of RIZ1. Abnormal expression of RIZ1 has been found to be associated with tumor invasion and malignancy.[4-10]

Our group has previously reported that RIZ1 expression level is lower in esophagus carcinoma than in adjacent noncancerous tissues[10], and is related to methylation of CpG islands[12]. In addition, by constructing human RIZ1 eukaryotic expression vectors to transfect human esophageal squamous cell carcinoma (ESCC) cell line TE13, we were able to report that upregulation of RIZ1 can recover tumor suppression activity and that treatment of cell line TE13 by methyltransferase inhibitor 5-aza-CdR reverses the methylation status of the promoter region[13]. In order to investigate RIZ1-mediated changes in gene expression of esophageal cancer, we compared the gene expression profile of TE13 cells transfected with RIZ1 with those of negative control cells. The resulting changes in oncogenicity were analyzed in vivo and by animal experimentation.

Materials and Methods

Ethics

The animal study proposal was approved by the Tianjin Medical University General Hospital Ethics Committee with the permit number: 2012-021. All mouse experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People’s Republic of China.

Cell culture and transfection

Human ESCC cell line TE13 was purchased from ATCC (Rockville; MD, United States) and cultured in RPMI-1640 (HEPES 4.76 g/NaCO₃ 2.0 g/RPMI-1640 10.4 g/ddH₂O 1000 mL) media supplemented with 10% new-born bovine serum, 2 mmol/L 1 × L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Life Technologies; NY, United States). Cells were maintained at 37°C in a humidified atmosphere with 5% CO₂. RIZ1 eukaryotic expression vector pcDNA3.1(+)/RIZ1 plasmid had been prepared previously and stored at −80°C. Cultivated TE13 cells were passaged in 12-inch orifice plates. Passaging was repeated every 2-3 d at 1:10 dilution, and cells were lifted by trypsin digestion. When the cells were at the log phase, they were transfected using the classical liposome method by adding 2 μL Lipofectamine 2000 (Life Technologies; NY, United States). Experimental and control groups were transfected with pcDNA3.1(+)/RIZ1 and pcDNA3.1(+), respectively. The media were changed after 6 h and the cells were washed in phosphate buffered saline (PBS), harvested and counted. After mixing with Trizol Remex Ex TaqTM (Taka). The following primer sets (10 μmol/L) were used: RIZ1, forward 5'-TCTGCTGTGACAAGACC3'-3', reverse 5'-GCATCATGCACATCCCATC3'-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5'-ACCAGAAGACTTGGAATGG3'-3', reverse 5'-TTCAGCTCAGGATGACCTT3'-3'. Amplification was carried out using LightCycler real-time polymerase chain reaction (PCR) system (Roche, United States), according to the manufacturer’s protocol. Each sample was run in triplicate for each gene. An initial denaturation step at 95°C for 5 min was followed by 40 denaturation cycles at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s. A solubility temperature curve assay was constructed and the RIZ1 and GAPDH Ct-values for each group were recorded and compared. The RIZ1 mRNA relative quantitation formula: (2^(-ΔΔCt) × 100%), was applied to evaluate whether transfection was successful. Duplicate detections were performed in triplicate, through 2^(-ΔΔCt), to calculate mean ± SD.

Microarray analysis

Total RNA was extracted using Trizol Reagent (Life Technologies; NY, United States). Quality control was achieved by utilizing the Agilent Bioanalyzer 2100 (Agilent Technologies; United States). Purification was achieved using the RNeasy mini kit and RNase-Free DNase Set (QIAGEN, Germany). Agilent’s Low Input Quick Amp Labeling Kit, one-color and full genome chip (4 × 44K, design ID: 014850) were used to amplify and mark the mRNA according to the manufacturer’s protocols. The eRNA was purified and conjugated using the RNeasy mini kit. Agilent’s Gene Expression Hybridization Kit with a sample quantity of 1.65 μg eRNA was employed for gene chip hybridization for 17 h in a hybridization oven at < 65°C and 10 rpm, following Agilent’s protocol. Slides were washed in staining dishes (Thermo Shandon, WJG | www.wjgnet.com 6171 October 7, 2013 | Volume 19 | Issue 37 |
Cultured cells were used for subsequent experimentation. Tissue from each group was homogenized in RIPA buffer (50 mmol/L Tris-HCl, pH 7.4; 150 mmol/L NaCl; 1% Nonidet P-40; 0.5% sodium deoxycholate; 0.1% SDS; 1 mmol/L EDTA; 1 mmol/L PMSF; 1 mg/mL Aprotinin). The supernatant was collected and protein concentrations were determined by a bicinchoninic acid (BCA) protein assay kit (Pierce; IL, United States); 30 μg of whole-cell lysate was separated on 8% SDS-PAGE gels, transferred to nitrocellulose (NC) membranes (Amersham Biosciences; New Jersey, United States), and immunoblotted with the following antibodies: anti-β-actin (ABCAM; United Kingdom), control; primary antibodies, 1:2000 dilution (ABCAM; United Kingdom); secondary antibodies Goat Anti-Mouse, 1:5000 dilution (ABCAM; United Kingdom). The films were analyzed by a PowerLook scanner (UMAX) and quantified by Image Quant software (GE; United States). The control experiments (TE13 cells; TE-13 cells transfected with blank plasmid) were treated by the same method. Relative expression of RIZ1 = gray value of RIZ1 protein/gray value of β-actin.

RESULTS

Transfection

The melting curve peaks for RIZ1 and GAPDH transcript products were at 80 °C and 82.5 °C, respectively (Figure 1), giving Ct-values for the two groups. The relative expression levels were calculated using the real-time PCR relative quantitative formula. RIZ1 gene expression levels were compared with SPSS v.13.0 statistical software. The results showed that the mRNA expression level in the experimental group was higher than in the control groups (Figure 2), indicating that transfection had been successful (P ≤ 0.01).

Alteration of gene expression profile

Table 1 gives the 2100 results for RIN ≥ 7.0 and 28S/18S ≥ 0.7, therefore qualifying the samples without degradation. The initial scanned single fluorescence chip data (Figure 3A) were standardized and converted to logarithmic values. A scatter plot was constructed with a two-dimensional rectangular coordinate plane (Figure 3B).

GO and microarray analysis

The SAS system was used for GO analysis of the differentially expressed genes and P ≤ 0.05 was considered to be statistically significant (Table 2). The microarray data showed that 2123 genes were differentially expressed in the pcDNA3.1 (+)/RIZ1 transfected cells with fold expression. One of these genes is RIZ1, which encodes a transcription repressor.
Transplantation of human esophageal cancer TE13 cells into nude mice was successful as shown in Figure 5. Tumor volumes were compared among the three groups (Figure 6A). The tumor growth rate curves revealed that tumor growth was slower in the pcDNA3.1(+)/RIZ1 group, with a shallower growth rate curve, than in the control group, suggesting that the RIZ1 gene may participate in multiple signaling pathways (P < 0.01), some of which changes > 2 (P < 0.05) compared to control samples. Of these, 960 genes were upregulated, of which 654 were known genes (1.70%, 654/38500) and 306 were unknown; 1163 genes were downregulated, of which 719 were known genes (1.87%, 719/38500) and 444 were unknown. Subsequent analyses were primarily carried out on annotated genes. The gene chip results were confirmed by RT-PCR (Figure 4).

**Pathway analysis**

Many of the identified genes are associated with cell development, virus replication supervision, costimulatory molecule, and immune system development. Further analysis indicated that the RIZ1 gene may participate in multiple signaling pathways (P < 0.01), some of which are given in Table 3.

**Transplantation and tumor growth**

Transplantation of human esophageal cancer TE13 cells into nude mice was successful as shown in Figure 5. Tumor volumes were compared among the three groups (Figure 6A). The tumor growth rate curves revealed that tumor growth was slower in the pcDNA3.1(+)/RIZ1 group, with a shallower growth rate curve, than in the control group.
Table 2  Gene ontology analysis of differentially expressed genes

| GO ID       | Name                        | Hits | Total | Percentage | P value |
|-------------|-----------------------------|------|-------|------------|---------|
| GO:0048468  | Cell development            | 81   | 832   | 9.74%      | 0.0288  |
| GO:0050792  | Regulation of viral         | 5    | 20    | 25.00%     | 0.0304  |
|            | reproduction                |      |       |            |         |
| GO:0031294  | Lymphocyte costimulation    | 3    | 8     | 37.50%     | 0.0397  |
| GO:0002520  | Immune system development   | 38   | 358   | 10.61%     | 0.0428  |

Table 3  RIZ1 pathway analysis (selected pathways)

| Name                      | Hits | Total | Percentage | P value | q     |
|---------------------------|------|-------|------------|---------|-------|
| Cytokine-cytokine         | 25   | 276   | 9.06%      | 0       | 0     |
| receptor interaction      |      |       |            |         |       |
| TGF-beta signaling        | 10   | 85    | 11.76%     | 3.00E-04| 5.00E-04|
| pathway                   |      |       |            |         |       |
| MAPK signaling            | 20   | 271   | 7.38%      | 2.00E-04| 5.00E-04|
| pathway                   |      |       |            |         |       |
| Pathways in cancer        | 20   | 328   | 6.10%      | 0.0018  | 0.0015|

Figure 4  Comparison between results from the gene chip and the reverse transcription-polymerase chain reaction. Out of the nine genes tested, eight yielded consistent results for both reverse transcription-polymerase chain reaction and gene chip. The five upregulated genes are CDK6, HOXB7, KITLG, APPL1, TGFβ2; and the three downregulated genes are CASP14, AIFM3 and NAIF1; however, in the case of service principal name (SPN), the reverse transcription-polymerase chain reaction results indicate upregulation, whereas the gene chip results indicate downregulation.

Figure 5  Photograph showing tumor development in a nude mouse, confirming successful transplantation of human esophageal cancer into an animal model.

DISCUSSION

Esophageal cancer is the world's most common cancer of the digestive system, with 70% of incidences occurring in China. China also has the highest incidence and mortality rates for both men and women. Recent statistics on morbidity and mortality for cancer patients show that esophageal cancer is the 6th most common form of cancer, and the 4th highest cause of cancer death-related in China[14-18]. Furthermore, the incidence rate is higher in rural areas than in urban areas. Esophageal cancer can be divided into two pathological types: ESCC and esophageal adenoid carcinoma. In China, ESCC accounts for 90% of esophageal cancers, in contrast to Western countries.

Treatment of esophageal carcinoma is a long-term project; however, by combining several different treatment types, the quality of life of the patients can be greatly improved. The purpose of our study is to enhance the understanding of ESCC development and mechanisms at the genetic level in order to advance clinical therapies. RIZ1 is one of the most effective tumor sup-
pression genes, therefore failure of RIZ1 expression can lead to the development of many forms of cancer [19-25].

Our group has carried out a number of studies on the RIZ1 gene [11,12]; however, further research is required. One key research area is the introduction of a foreign gene into the tumor tissue, which is then able to express stably; eukaryotic expression vector is an ideal choice. The liposome mediated method was adopted because it has the advantages of high transfection efficiency, low immunogenicity, simple manipulation, and can be applied to a wide variety of cells.

We constructed the RIZ1 gene eukaryotic expression vector pcDNA3.1(+)/RIZ1 using an established molecular biology technique; empty plasmid, pcDNA3.1(+), was used as a negative control. After transfection into ESCC cell line TE13, in vivo experiments and gene chip analyses were carried out, as described in Materials and Methods. Our results showed that the xenograft in nude mice had a slower growth rate, and lower tumor volume and mass, in the pcDNA3.1(+)/RIZ1 group than in the blank control and pcDNA3.1(+) groups. This suggests that RIZ1 has a restraining effect on ESCC tumor growth. In contrast, the growth curves for the blank control and pcDNA3.1(+) groups were approximately parallel, indicating that these groups had no restraining effect.

Secondly, after transfection, the gene expression profile of the cell line TE13 underwent extensive changes, with a total of 2123 differentially expressed genes, including 1163 downregulated and 960 upregulated. We found that many of these genes are involved in cell development, lymphocyte costimulatory, immune system development, and interestingly, in the supervision of viral replication. This is consistent with the results from Dąbrowski et al [13] who reported that low-risk type human papilloma virus may be one of the auxiliary activated or carcinogenic factors in ESCC occurrence and development. Vaiphei et al [26] also found that the infection rates of human papilloma virus in ESCC patients was as high as 87%, especially in patients with two or more types of phenotypic mixed infection. Persson et al [27] reported that HIV infection increases the risk of esophageal cancer. All these reports indicate that the occurrence and development of esophageal cancer may be related to virus infection; however, the pathophysiological mechanisms are poorly understood and require further research.

In conclusion, we propose that the occurrence of esophageal cancer is a consequence of widespread alterations in gene expression, involving multiple functions and signaling pathways with roles in tumor development, some of which are synergistic or antagonistic. We also speculate that the most probable signaling pathways in ESCC affected by these genes are the cytokine receptor interaction and transforming growth factor pathways.
Therefore, we recommend that future research should be directed towards better understanding of the relationship between the RIZ1 gene and ESCC and the mechanism and role of virus infection in ESCC occurrence and development.

COMMENTS

Background
Esophageal cancer is one of the most common forms of cancer in the world and is a leading cause of cancer deaths in many developing countries. China is a country with a high incidence of esophageal cancer, and the pathological type is mainly the squamous cell carcinoma, which is different from the Western countries where adenocarcinoma is reported to be the main pathological type. To date, the mechanisms of esophageal cancer are unclear. Tumor occurrence and development are regulated by a variety of oncogenes and tumor suppressor genes, including the putative tumor suppressor gene, retinoblastoma protein-interacting zinc finger gene 1 (RIZ1).

Research frontiers
RIZ1 expression is lower in esophage carcinoma and is related to methylation of CpG islands. In addition, by constructing human RIZ1 eukaryotic expression vectors to transfect human esophage squamous cell carcinoma (ESCC) cell line TE13, the authors were able to report that upregulation of RIZ1 can recover tumor suppression activity and that treatment of cell line TE13 by methyltransferase inhibitor 5-aza-CdR reverses the methylation status of the promoter region.

Innovations and breakthroughs
In order to investigate RIZ1-mediated changes in gene expression of esophageal cancer, the authors compared the gene expression profile of TE13 cells transfected with RIZ1 with that of negative control cells. The resulting changes in oncogenicity were analyzed in vitro and by animal experimentation. They found that the development and progression of esophageal cancer are related to the inactivation of RIZ1. In addition, virus infection may also be an important factor.

Applications
RIZ1 is one of the most effective tumor suppression genes, therefore failure of RIZ1 expression can lead to the development of ESCC, which is expected to be a molecular biological parameter for early diagnosis.

Peer review
This paper reports that the development and progression of esophageal cancer is relevant to the inactivation of RIZ1. In addition, virus infection may also be an important factor.

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