Next Generation Sequencing for Potential Regulated Genes and Micro-RNAs of Early Growth Response-1 in the Esophageal Squamous Cell Carcinoma

Yen-Chiang Tseng1,2,3, Chih-Wen Shu4,5, Hui-Min Chang6, Yi-Hsuan Lin7,8, Yen-Han Tseng9,10, Han-Shui Hsu3,10,11, Yih-Gang Goan1,10, Ching-Jiunn Tseng2,6

Accepted: 21 September 2022 / Published online: 7 October 2022 © The Author(s) 2022

Abstract
Esophageal cancer has a poor prognosis due to its aggressiveness and low survival rate. In East Asia, esophageal squamous cell carcinoma (ESCC) outnumbers esophageal adenocarcinoma (EAC). The ESCC patients still have high mortality despite modern surgical resection and neoadjuvant treatment. Determining patient and outcome prognostic factors is critical in ESCC treatment. In esophageal cancer, early growth response-1 (Egr-1) is a tumor suppressor gene, but the mechanism and associated genes are unknown. The study utilizes RNA interference method, the platform of Next Generation Sequencing (NGS) and bioinformatics analysis to investigate the influences after the Egr-1 gene slicing on the ESCC cells. The heat maps of differentially expressed mRNA and microRNAs were analyzed using the algorithm, Burrows-Wheeler Aligner. The study showed that the expression of 51 mRNA and 26 microRNAs have significant changes in ESCC cells after Egr-1 knockdown. The KEGG enrichment analysis linked Egr-1-regulated genes and microRNAs. Egr-1 interactions with these genes and microRNAs may be important in tumor progression. In conclusions, this study provided the transcriptome patterns and relating pathway analysis for Egr-1 knockdown in ESCC cells. The mRNA and microRNAs altered by Egr-1 gene silencing might provide key information in the treatment of ESCC.

Keywords EGR1 · Tumor suppressor · Esophageal carcinoma

Chih-Wen Shu
cwshu@g-mail.nsysu.edu.tw
Ching-Jiunn Tseng
cjtseng@vghks.gov.tw

1 Division of Thoracic Surgery, Department of Surgery, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan
2 Institute of Clinical Medicine, National Yang Ming Chiao Tung University, Taipei, Taiwan
3 Division of Thoracic Surgery, Department of Surgery, Taipei Veterans General Hospital, Taipei, Taiwan
4 Institute of BioPharmaceutical Sciences, National Sun Yat-Sen University, No. 70, Lianhai Rd., Gushan Dist, Kaohsiung 80424, Taiwan
5 Department of Biomedical Science and Environmental Biology, Kaohsiung Medical University, Kaohsiung 80708, Taiwan
6 Department of Medical Education and Research, Kaohsiung Veterans General Hospital, No. 386, Dazhong 1st Rd., Zuoying Dist, Kaohsiung 81362, Taiwan
7 Department of Family Medicine, School of Medicine, National Yang Ming Chiao Tung University, Taipei, Taiwan
8 Department of Public Health, College of Public Health, National Taiwan University, Taipei, Taiwan
9 Department of Chest Medicine, Taipei Veterans General Hospital, Taipei, Taiwan
10 School of Medicine, National Yang Ming Chiao Tung University, Taipei, Taiwan
11 Institute of Emergency and Critical Care Medicine, National Yang Ming Chiao Tung University, Taipei, Taiwan
1 Introduction

Esophageal cancer is associated with poor survival rate despite surgical resection. Two main histological subtypes, esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), have significant differences in epidemiology, etiology, and treatment response. In East Asia and Africa, ESCC is the predominant subtype. Many clinicopathological variables, including the depth of tumor invasion, lymph node involvement, lymphovascular invasion, intramural metastasis, and the stage of disease, have been examined to predict the prognosis. Multiple molecular changes have also been investigated to elucidate the mechanism of ESCC tumorigenesis [1–3]. In recent years, neoadjuvant treatment, including neoadjuvant chemotherapy (nCT) or neoadjuvant chemoradiotherapy (nCRT), followed by esophagectomy, has become the standard strategy for resectable locally advanced esophageal cancer due to the survival benefit. Moreover, the pathological response after nCT or nCRT has been demonstrated to be independently associated with overall survival [4, 5]. Therefore, it is important to set up a new system to predict the pathological response and survival rate.

The early growth response-1 (Egr-1) is a zinc-finger transcription factor of 59 Kilodaltons. Egr-1 is involved in the regulation of cell growth and differentiation in response to signals, such as mitogens, growth factors, and stress stimuli [6]. Analysis of certain human tumor cells and tissues indicate that Egr-1 acts as a tumor suppressor [7–9]. Egr-1 suppress the function of 4E-BP1, which in turn sequester eIF4E and lead to rapamycin insensitivity. Gao et al. showed that miRNA-191 modulates Egr-1 and the prognosis of ESCC [10]. Zhao et al. showed that Egr-1 represses ESCC through ERK1/2 signaling pathway [11]. Other studies revealed miRNAs are involved in the origin and development stage of ESCC as well as chemoresistance [12–15]. These articles indicate that Egr-1 play important roles in ESCC by controlling cell growth, proliferation, differentiation, and angiogenesis both epigenetically and genetically. However, variable miRNAs, together with different miRNAs-mRNAs regulatory systems, lead to complex outcomes that require further investigation [16].

Our preliminary data revealed that Egr-1 is an important tumor suppressor which highly correlates with chemoresistance in ESCC (in pressing). The Egr-1 could play an important role as a tumor suppressor as well as influencing the chemotherapeutic effects. Therefore, we investigated the miRNAs linking in the Egr-1 as well as explore the relationship between Egr-1 and these miRNAs to explore the miRNAs-mRNAs regulation in ESCC.

2 Materials and Methods

2.1 Cell Culture

Esophageal cancer cell line, CE81T, were obtained from Dr. Cheng-Po Hu at Taipei Veterans General Hospital and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen-Gibco, Carlsbad, USA), with 10% heat-inactivated fetal bovine serum (Biological Industries, Kibbutz Beit-Haemek, Israel), 100 U/mL penicillin (Invitrogen-Gibco, Carlsbad, USA), 1% MEM non-essential amino acids (NEAA) and 100 µg/mL streptomycin (Invitrogen-Gibco, Carlsbad, USA), at 37 °C in a humidified 5% CO₂ atmosphere. Cells were grown in Corning tissue culture-treated plastic (Corning, Inc., Corning, USA).

2.2 siRNA Knockdown

siRNA-mediated EGR-1 knockdown was carried out in the CE81T cell line to observe the cell growth and viability of ESCC tumor cells. The EGR-1 siRNA oligos pool (1:5′-GAUGAACCGCAAGGCAUA-3′; 2:5′- CGACAGCAUGCACAUUAC-3′; 3:5′-GGACAUGACGACACCUUUU-3′; 4:5′-GACCUGAAGGCCCCUAAUA-3′) were synthesized by Genomics BioScience and Technology Co., Ltd. (Taipei, Taiwan). All transient transfections of the siEGR-1 oligos pooled at a final concentration of 10 nM were accomplished with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocols. Esophageal cancer cells were seeded into six-well flat-bottom plates of 3×10⁵ cells per well-containing 1 mL of the medium. The siRNA oligonucleotides and RNAiMAX reagent were separately diluted by 100 µL of the opti-MEM medium and mixed, the mixture was then incubated for 15–20 min. The cells were incubated with the transfection medium overnight at 37 °C in a humidified atmosphere of 5% CO₂. Cells were incubated for 24, 48, or 72 h before harvesting. Non-silencing control (NSC) was used at the same concentration of siRNAs.

2.3 RNA Purification and Reverse Transcription

Cellular mRNA was extracted by TRIzol® following the protocol provided by the manufacturer. Briefly, culture media were removed and 1 mL TRIzol® is added into the culture plate. The plate is shaken gently to make TRIzol® lyse all the cells and a pipetman was used to pipet the cell lysate up and down several times to homogenize the mixture. After incubating 5 min in room temperature, the whole content was moved to a new 1.7 mL centrifuge tube by a pipetman. Chloroform of 0.2 mL was added into the centrifuge tube.
Fig. 1  NGS was applied on the CE81T cells after Egr-1 knockdown for 48 or 72 h. The Venn diagram identified A 51 mRNAs and B 26 microRNAs with the persistent effect after gene knockdown. Knockdown is abbreviated as KD.
and securely capped the tube. The tube was inverted several
times to homogenize the solution. After incubating 3 min, the
samples were centrifuged for 15 min at 12,000 × g at 4°C.
The upper layer of centrifugated samples were transferred
into a new 1.7 mL centrifuge tube with 0.5 mL isopropanol.
Solutions were centrifuged again for 10 min after 10 min
incubation and the supernatants were discarded. Precipitated
RNA was washed by 75% ethanol and centrifuged at 7500
× g, 4 °C for 5 min. Finally, RNA in tube was dried at room
temperature and solubilized by RNase-free water with proper
volume. RNA solution was stored under –80 °C avoiding
freeze-thaw cycle. RNA content and purity was measured by
Nanodrop microvolume spectrophotometer (Thermo-Fisher).

For reverse transcription, 2 µg of total RNA was con-
verted to cDNA using reverse transcription kit (Applied
Biosystems, Foster City, CA, USA). The procedure was the
same as manual instructed.

2.4 Next Generation Sequencing

The human esophageal cancer cell line CE48T were
sequenced to an average sequencing coverage of at least
300× on Illumina Hiseq 4000 platform (Illumina, San
Diego, CA). The sequencing data were first demultiplexed
by bck2fastq and then subjected to Trimmomatic for FASTQ
file quality control (QC). Leading/trailing low quality (Phred
score below 15) reads or N bases were removed. PCR dupli-
cates were removed by Picard. Qualified reads were mapped
to the reference human genome hg19 with Burrows-Wheller
Aligner (BWA-mem, v0.7.12). Genome Analysis Toolkit
(GATK 3.4.0) was used for local realignment around indels
and base quality score recalibration. ADTEx was used to
identify copy number variations (CNVs) using a normal
human HapMap DNA sample NA18535. The log2 ratio cut-
off value was set at ± 0.6, which excluded target genes with
copy number change between 1.5 and 0.65 fold.

2.5 Bioinformatic Analysis

The pathway related to the target genes were performed by
KEGG. Target miRNA were predicted by miRbase (https://
www.mirbase.org/) and Rfam (https://rfam.xfam.org/).

3 Results

3.1 Screening of Genes and MicroRNAs
in the Transcriptome of the Esophageal Cancer
Cell Line After Egr-1 Knockdown

To explore the mRNAs and microRNAs with overlapped
expression profiles, we compared the NGS analysis between

---

Fig. 2 The heat maps of the A 51 mRNAs and B 26 microRNAs
identified on the CE81T cells after Egr-1 knockdown for 48 and 72 h.
CE81T cells after Egr-1 knockdown 48 or 72 h. The 51 genes (Fig. 1A) and the 26 microRNAs (Fig. 1B) with overlapped expression patterns were determined in the NGS analysis. The cluster analysis of differentially expressed genes in the CE81T cells for the post Egr-1 knockdown was analyzed on microarray and bioinformatic analysis. Figure 2 are the heat maps of the 51 genes (Fig. 2A) and the 26 microRNAs (Fig. 2B) derived from the Egr-1 knockdown CE81T cells for 48 or 72 h. The relative fold changes of 51 genes and 26 microRNAs on the CE81T cells for the post Egr-1 knockdown 48 or 72 h was shown in Fig. 3.

3.2 Bioinformatic Analysis for the Functions of Genes and MicroRNAs in the CE81T Cells After Egr-1 Knockdown

KEGG analysis of the 51 genes in CE81T cells after Egr-1 knockdown showed that they mainly related to organismal systems, metabolism, human diseases, genetic information processing and environmental isolation processing (Fig. 4). The rich factor showed that the genes were mainly enriched in ribosome, proteoglycans in cancer, and glucagon signaling pathway (Fig. 5); while the miRNAs were mainly enriched with viral myocarditis, phagosome, MAPK signaling pathway, focal adhesion, and cell adhesion molecules (Fig. 5). We also screened out three microRNAs which interacted with the seven mRNAs with persistent effect in 48 and 72 h after knockdown of Egr-1 (Table 1).

4 Discussion

This is a pilot study to apply NGS to identify the interaction between mRNAs and microRNAs after EGR-1 knockdown in ESCC cells. We analyze the transcriptome which include mRNAs and small non-coding RNAs like miRNAs. Two expression pattern, Egr-1 knockdown for 48 or 72 h in ESCC
cells, were compared and analyzed by bioinformatic system. The co-expression networks and predicted candidate molecules could be used to find the precision medicine for the chemotherapeutic and therapeutic targets.

Four mRNAs with the same changing pattern after Egr-1 knockdown for 48 or 72 h stand out in Fig. 3A. The expression of NLGN4X is down-regulated, but it is increased in TENM4, NPR3, and IGFN1. NLGN4X is a human specific gene for neuroligin production. Neurilgins, a family of cell adhesion molecules, are essential for synapse specification and maturation [17]. The product of TENM4 is a member of teneurin family. Teneurins can bind with latrophilin and FLRT, and direct synapse growth and formation [18, 19]. NPR3 mediates natriuretic peptides degradation, was reported to act as a tumor suppressor or promoter in some types of cancer [20, 21]. IGFN1 is a multidomain protein with more than 15 transcript variants. Aberrant splicing may lead to intronic G-quadruplex be synthesized, and lead to cancer or neurodegenerative disorder [22]. In these four genes, NLGN4X and TENM4 are adhesion molecules. Mis-regulated adhesion molecules may lead to structural deformation which may benefit cancer progression. Since there are few reports regarding the role of these four genes in esophageal cancer cell, the meaning of Egr-1 controlled gene expression still needs further studies.

Three miRNAs were sorted out with interesting interactions and worth of further research (Table 1). miRNA-2110, an onco-suppressor with the ability to suppress breast cancer and neuroblastoma [23, 24], was down-regulated after Egr-1 knockdown for 48 and 72 h. This may indicate that Egr-1 control the fate of ESCC cells by regulating the expression of miRNA-2110. After short searching, majority of the genes controlled by miRNA-3615 and NovelmiRNA-74 are for structural meaning. Those genes and two miRNAs may help to stabilize an environment which is favored by the fate of ESCC cells. Since both miRNA-3615 and miRNA-74 have different expression patterns in Egr-1 knockdown for 48 or 72 h, the real functions and effects of those two miRNAs will need more strategies to figure out.

We conducted this pilot study in CE81T cell only, which might be a problem in generalizing the results in all the ESCC cases. CE81T cell derived from the well differentiated squamous cell carcinoma of esophagus from a 57-year-old male in Taiwan. It is widely used in the research of signal transduction, apoptosis, and even pharmaceutic field, especially in Taiwan. Our results should be confirmed in other ESCC cell lines derived from human like TE-1, TE-2, and...
Fig. 5  KEGG pathway enrichment bubble plot of the A 51 mRNAs and the B 26 microRNAs identified in CE81T cells after Egr-1 knockdown with persistent effect. The dot size displayed the number of genes, whereas the dot color indicated the Q-Value of the rich factors.
Table 1 The miRNAs-mRNAs interaction identified by bioinformatics analysis

| miRNA_ID  | mRNA_ID       | GeneID       | Chr | Start  | End    | Strand | Gene Symbol |
|-----------|---------------|--------------|-----|--------|--------|---------|-------------|
| hsa-miR-2110 | ENST00000239938 | ENSG00000120738 | 5   | 1.38E+08 | 1.38E+0.8 | +      | EGRI        |
| hsa-miR-3615 | ENST00000278550 | ENSG00000149256 | 11  | 78,363,876 | 79,151,992 | –      | TENM4       |
| NovelmiRNA-74 | ENST00000345356 | ENSG00000111799 | 6   | 75,794,042 | 75,915,767 | –      | COL12A1     |
| hsa-miR-3615 | ENST00000398097 | ENSG00000188158 | X   | 17,393,543 | 17,754,114 | +      | NHS         |
| NovelmiRNA-74 | ENST00000419587 | ENSG00000232382 | 3   | 97,540,884 | 97,594,132 | +      | CRYBG3      |
| hsa-miR-3615 | ENST00000467148 | ENSG00000130589 | 20  | 62,189,439 | 62,205,592 | –      | HELZ2       |
| NovelmiRNA-74 | ENST00000526600 | ENSG00000187079 | 11  | 12,695,969 | 12,966,298 | +      | TEAD1       |

Three main microRNAs interacted with seven mRNAs after Egr-1 knockdown and had persistent effect in 48 and 72 h. The hsa-miR-2110 interacted with EGR1. The hsa-miR-3615 interacted with TENM4, NHS and HELZ2. NovelmiRNA-74 interacted with TEAD1, CRYBG3 and COL12A1.

CRL-3239. The confirmed results can then serve as the foundation for the studies of ESCC treatment.

5 Conclusion

There are tons of information collected after NGS sequencing. We need further evaluations and experiments to confirm the interactions between microRNAs and mRNAs. Various ESCC cell lines will be also included to determine the gene regulation by Egr-1 is consistent among different ESCC cell lines. Nevertheless, the application of NGS to identify related microRNAs as well as mRNAs after EGR-1 knockdown would be an initial point for novel prognostic factors of ESCC.

Funding The work was supported by the Ministry of Science and Technology MOST (108-2320-B-110-008-MY3) and the Kaohsiung Veterans General Hospital (VGHKS108-075).

Declarations

Conflict of interest The authors declare that there are no competing interests associated with the manuscript.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. Material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

References

1. Abnet CC, Arnold M, Wei WQ (2018) Epidemiology of esophageal squamous cell carcinoma. Gastroenterology 154(2):360–373
2. Hsu PK, Li AF, Wang YC et al (2008) Reduced membranous beta-catenin protein expression is associated with metastasis and poor prognosis in squamous cell carcinoma of the esophagus. J Thorac Cardiovasc Surg 135(5):1029–1035
3. Rustgi AK, El-Serag HB (2014) Esophageal carcinoma. N Engl J Med 371(26):2499–2509
4. Meredith KL, Weber JM, Turaga KK et al (2010) Pathologic response after neoadjuvant therapy is the major determinant of survival in patients with esophageal cancer. Ann Surg Oncol 17(4):1159–1167
5. Wang BY, Wu SC, Chen HC et al (2019) Survival after neoadjuvant chemoradiotherapy and oesophagectomy versus definitive chemoradiotherapy for patients with oesophageal squamous cell carcinoma. Br J Surg 106(3):255–262
6. Krones-Herzig A, Mittal S, Yule K et al (2005) Early growth response 1 acts as a tumor suppressor in vivo and in vitro regulation of p53. Cancer Res 65(12):5133–5143
7. Baron V, Adamson ED, Calogero A, Ragona G, Mercola D (2006) The transcription factor Egr1 is a direct regulator of multiple tumor suppressors including TGFbeta1, PTEN, p53, and fibronectin. Cancer Gene Ther 13(2):115–124
8. Rolli-Derkinderen M, Machavoine F, Baraban JM, Grolleau A, Beretta L, Dy M (2003) ERK and p38 inhibit the expression of 4E-BP1 repressor of translation through induction of Egr-1. J Biol Chem 278(21):18859–18867
9. Yun S, Vincelette ND, Knorr KL et al (2016) 4EBP1/c-MYC/PUMA and NF-kappaB/EGR1/BIM pathways underlie cytotoxicity of mTOR dual inhibitors in malignant lymphoid cells. Blood 127(22):2711–2722
10. Gao X, Xie Z, Wang Z, Cheng K, Liang K, Song Z (2017) Overexpression of miR-191 predicts poor prognosis and promotes proliferation and invasion in esophageal squamous cell carcinoma. Yonsei Med J 58(6):1101–1110
11. Zhao Y, Xia Q, Liu Y et al (2019) TCF7L2 and EGR1 synergistic activation of transcription of LCN2 via an ERK1/2-dependent pathway in esophageal squamous cell carcinoma cells. Cell Signal 55:8–16
12. Harada K, Baba Y, Ishimoto T et al (2016) The role of microRNA in esophageal squamous cell carcinoma. J Gastroenterol 51(6):520–530
13. Qin G, Yang L, Ma Y, Liu J, Huo Q (2019) The exploration of disease-specific gene regulatory networks in esophageal carcinoma and stomach adenocarcinoma. BMC Bioinform 20(Suppl 22):717

14. Sakai NS, Samia-Aly E, Barbera M, Fitzgerald RC (2013) A review of the current understanding and clinical utility of miRNAs in esophageal cancer. Semin Cancer Biol 23(6 Pt B):512–521

15. Wang T, Gu J, Li Y (2014) Inferring the perturbed microRNA regulatory networks from gene expression data using a network propagation based method. BMC Bioinform 15:255

16. Wang J, Yu P, Luo J, Sun Z, Yu J, Wang J (2021) Transcriptomic and microRNA Expression Profiles Identify Biomarkers for Predicting Neo-Chemoradiotherapy Response in Esophageal Squamous Cell Carcinomas (ESCC). Front Pharmacol 12:626972

17. Qin L, Guo S, Han Y, Wang X, Zhang B (2020) Functional mosaic organization of neureligins in neuronal circuits. Cell Mol Life Sci 77(16):3117–3127

18. Sando R, Jiang X, Südhof TC (2019) Latrophilin GPCRs direct synapse specificity by coincident binding of FLRTs and teneurins. Science 363(6429):eaav7969

19. Ziegler A, Corvalán A, Roa I, Brañes JA, Wollscheid B (2012) Teneurin protein family: an emerging role in human tumorigenesis and drug resistance. Cancer Lett 326(1):1–7

20. Gu L, Lu L, Zhou D, Liu Z (2018) Long noncoding RNA BCYRN1 promotes the proliferation of colorectal cancer cells via Up-regulating NPR3 expression. Cell Physiol Biochem 48(6):2337–2349

21. Li S, Guo R, Peng Z et al (2021) NPR3, transcriptionally regulated by POU2F1, inhibits osteosarcoma cell growth through blocking the PI3K/AKT pathway. Cell Signal 86:110074

22. Verma SP, Das P (2018) Novel splicing in IGFN1 intron 15 and role of stable G-quadruplex in the regulation of splicing in renal cell carcinoma. PLoS One 13(10):e0205660

23. Zhang X, Li F, Zhou Y et al (2021) Long noncoding RNA AFAP1-AS1 promotes tumor progression and invasion by regulating the miR-2110/Sp1 axis in triple-negative breast cancer. Cell Death Dis 12(7):627

24. Zhao Z, Partridge V, Sousares M et al (2018) MicroRNA-2110 functions as an onco-suppressor in neuroblastoma by directly targeting Tsukushi. PLoS One 13(12):e0208777

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.