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miR-124 Inhibits Invasion and Metastasis in Esophageal Carcinoma by Targeting STAT3

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Abstract

Esophageal carcinoma is a high morbidity and mortality, malignant gastrointestinal tumour. Its presence and cycle of growth includes the expression imbalance within multiple genes. MicroRNA is the sort of particular-trapped non-coding trivial RNA which extensively engaged concerning post-transcriptional gene regulation and is involved concerning enhancement and occurrence of fatal cancers. The work explores role of miR-124's representation, regulation concerning esophageal carcinoma. RT-PCR had been applied for the discovery miR-124 expression concerning the tissues and cell connections of the esophageal carcinomas. A Link within miR-124 manifestation concerning esophageal carcinoma nerves, clinicopathological parameters and esophageal carcinoma prognosis was examined. CCK-8 and Transwell assays are used for detecting cell propagation, movement, cancer cell incursion. Bioinformatic analysis utilized to predict the downstream objective genes of miR-124 and verified by double-luciferase assay; and also verified Western blot 's influence of miR-124 on STAT3. MiR-124 demonstration concerning esophageal cancer muscles and cell lines was significantly down-regulated. The level of manifestation of miR-124 had been appreciably connected having phase TNM, depth of invasion and metastasis of lymph nodes, and cycle of survival. The expression level miR-124 had been the impartial threat aspect to diseased persons concerning esophageal cancer. MiR-124 overexpression significantly diminished the cell production, resettlement, and incursion of oesophageal cell lines. The double luciferase test demonstrated miR-124 specifically pursuing STAT3 and inhibiting expression of STAT3.

Conclusion: MiR-124 expression in the esophageal carcinoma is down-regulated which affects the degree of tumor progression and prognosis. MiR-124's potential mechanism is targeting and expressing STAT3.

Key words: miR-124; pathological parameters; esophageal cancer; STAT3.

1. Introduction

Esophageal carcinoma (EC) is a widespread tumor within the digestive tract. This is identified worldwide in the sixth high cancer death ratio and also as an esophageal quamous cell carcinoma..

With medical level advancement, surgical treatment, radiotherapy and chemotherapy and biological immunotherapy are used concerning dealing of esophageal cancer, which has increased effectiveness of the treatment of esophageal cancer, but some patients are still unable to achieve the anticipated impact. Cancer cells in the body have undergone invasion and metastasis, resulting in rapid tumor growth and poor patient prognosis, particularly to diseased persons with advanced

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esophageal cancer. To prevent and treat esophageal cancer, therefore, this seems crucial for analysis the molecular mechanism for esophageal cancer and to find new markers for the diagnosis and treatment of esophageal cancer.

MicroRNAs (miRNAs) have been the group of highly conserved small RNAs that participate in human pathological processes by targeting the 3'UTR of downstream objective genes based at the concept of base-pairing pairing so as to control target gene expression[1]. Most of the miRNAs found in the cells, and some are in the bloodstream or extracellular environment [2]. Most miRNAs have been found to be expressed uniquely in tumor tissues recently, resulting with prevalence and growth of tumours. Clinical studies are shown that miRNAs possess potential as early tumor diagnosis and prognosis indicators.

MiR-124 is a sort of plentiful miRNA in the brain. It has been discovered in recent years that it is expressed differently in many malignant tumors, especially digestive tract tumors, and participates in tumor cell proliferation, differentiation, development, stress and death. Nevertheless, some related work focuses mainly on gastric cancer, colorectal cancer, and [3~4] cholangiocarcinoma. There are few reports on miR-124 expression and its biological function in oesophageal cancer. Exploring miR-124's regulatory mechanism in oesophageal cancer can thus provide some new insights and solutions to improve the therapeutic effect of oesophageal cancer and prolong the life cycle of patients with oesophageal cancer.

The conducted work reveals the appearance of miR-124 concerning tumor muscles and contiguous ordinary muscles of diseased persons concerning esophageal cancer, and examined the influence of miR-124 expression on clinicopathological parameters and prognosis, and investigated the experimental worth of miR-124 differential countenance. Moreover, miR-124 demonstration had been observed in the esophageal cancer cell lines and the effect of miR-124 on the production, resettlement and incursion of oesophageal cancer cells was analyzed. The target gene was predicted to be STAT3, confirmed via dual-luciferase assay. Western blotting had been employed for the discovery miR-124's effect on expression of STAT3 protein.

2. Materials and procedures

Clinical samples our hospital reported 48 cases of oesophageal tissue specimens and standard neighboring cancer specimens. 3 senior pathologists have diagnosed all of the patients. The patient did not have any significant medical history, such as other cancers, and did not undergo radiation therapy, chemotherapy or any anti-tumor treatment prior to surgery. Upon discharge the patients were interviewed frequently. The committee for ethics of hospital accepted the conducted work and all diseased persons gave their signature on the letter provided by the committee for agreement. Samples were all fresh samples and were put in and deposited immediately in liquid nitrogen.

Cell cuture

ATCC, USA, purchased esophageal cancer EC-1, Eca109, EC9706 cells and human normal esophageal squamous cell NEECs. All esophageal cancer cells were grown in an RPMI average covering 10 percent fetal bovine serum and grown within carbon dioxide incubator at 37 ° C, 5 percent CO2, cell culture method had been altered after couple of days.

RNA isolation and RT-PCR of miR-124

After gathering cells in the logarithmic growth phase, and Trizol method purified total RNA from the liver cancer cells and tissues. Nanodrop 2000 spectrophotometer measured the concentration of the extracted RNA. Reverse transcription was done with a reverse transcription kit to get cDNA. The obtained cDNA was subjected to RT-PCR reaction by SYBR Green method, and the conditions of reaction were 95 ° C for 10 min; next 45 cycles of 95 ° C, 30s, 55 ° C. 30s. 30s. Upon completion of the reaction, the melting curve was evaluated for confirmation of the priming specificity. As an internal control, the expression level of the housekeeping gene GAPDH was used, and the target miRNA was quantitatively analyzed by some tt.

For miR-124 the introductions is suggested:

5'-GCTAAGCAACTCCGCGGT-3'; reverse: 5'-GTGCAGTTGAGACCGAGG-3'; internal reference primer sequence: 5'-ATTATATGGACCTGATTATACT-3'; reverse: 5'-ACGCTTCGCGGTATGCGCGTGTC-3 '

Cell transfection

Obtain good growth of oesophageal cancer cells, adjust cell density to 4105 cells / well, seed cells in 96-well plates, and transfected having miR-124 mimics, miR-124 mimics for each the LipfectamineTM 2000 transfection kit instructions. Regulator, miR-124 inhibitor and miR-124 inhibitor regulator plasmid were mixed with sufficient amount of cultivation solution, and then diluted Lipofectamine 2000 was applied to each well, carefully blended, and put at 37 ° C, 5% CO 2 after transfer. The cells were cultivated in an incubator of constant temperature, and RT-PCR verified the results of the transfection.

Cell proliferation assays

CCK-8 assay detected the growth rate of the cells, seeding the cells within logarithmic development process into 96-well plates (6bis 1103 cells / well), and adding 10 µl of CCK-8 to each well. At 0h, 12h, 24h, 36h and 48h of the 4 groups of cells, the strength absorbance at A450 nm was determined and the growth curve was drawn according to the results, and three replicate wells were set for each sample.

Cell migration and invasion assays

Matrigel gel was diluted on ice with a serum-permitted method by supplying Transwell space upper chamber, where Matrigel did not cover the cell migration assay. Four cell groups were made into cell suspension and supplemented uniformly at a density of 1.5105 cells / well within the Transwell upper chamber. In Transwell lowest chamber 600µl of the crop medium was added and placed at 37 ° C, 5 % CO2. Each group was set to 3 wells to replicate. The chamber was removed after 48 hours, and the upper chamber cells were wiped away by taking gauze. The cells on the lowest slot were washed 3 times with PBS buffer, fixed for 20 min with 95 % ethanol and painted for 20 min through 0.4% trypan blue, and examined under an inverted microscope.

Bioinformatics Analysis

Target gene prediction web sites TargetScan and miRanda were used to analyze miR-124 target genes, and then double luciferase assay utilized for the validity of required genes.

Dual luciferase assay

In six plates , cells had been sown and for this purpose, luciferase reporter had been co-transfected with a different gene group and grown overnight in an incubator at 37 ° C. Double-Luciferase Reporter Assay System utilized for calculating hRluc luciferase movement with hLuc / hRluc luciferase activity as the reference. For each sample three replicates are provided.

Western blotting

After 48 hours of transfection, washing the cells was needed and it had been through PBS bulwark thrice. After air-drying, total protein was extracted from different cells from cell lysate, and BCA assay detected the concentration of the extracted protein. Protein with a protein content of 40 µg per well was taken for 12 per cent SDS protein electrophoresis. The protein on the gel was transferred to the PVDF membrane, and a 1:1000 diluted primary antibody was added. A 1:5000 secondary antibody was added overnight after incubation at 4 ° C and kept at room temperature for 2 h. DAB color was applied, images were taken using an electrophoresis imager for the Bio-Rad gel, and image analysis was carried out using Quantity One software.

Statistical analysis

By the help of software the program SPSS 22.0, the facts had been examined .The data was revealed like mean ± standard deviation (x±s). In contrast between two classes the LSD-t assessment utilized. One-way variance investigation has been exploited for comparison within sets. Its numerical value is $P<0.05$.

3. Results

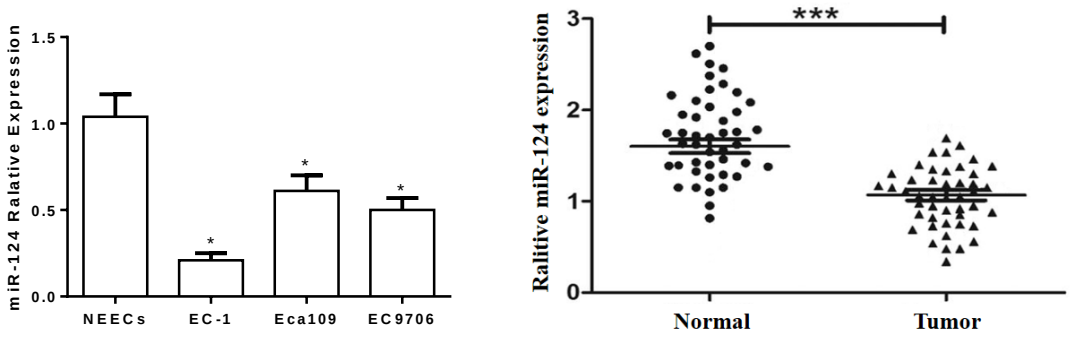
1. RT-PCR detection of miR-124 expression

Within oesophageal cancer tissues, the relative countenance of miR-124 had been expressively lesser as compared to neighboring natural tissues ($P<0.05$). Likewise, in the three lineages of esophageal cancer cells, appearance rates of miR-124 had been also expressively minor. Within regular oesophageal squamous cell carcinoma, as shown in table 1, miR-124 down-controlled within oesophageal cancer

2. Relationship between miR-124 expression and clinicopathological parameters in patients with esophageal cancer

We divided 48 diseased persons with esophageal cancer into miR-124 elevated appearance set as well as little appearance set with accordance to median relative expression of miR-124 within oesophageal cancer nerves. Statistical analysis showed that level of expression of miR-124 within diseased persons

through esophageal cancer and within diseased persons concerning phase TNM, extent of incursion and lymph node metastasis were significantly associated, but there was no significant gender , age and tumor differentiation correlation and it is revealed through figure 1.



(A) (B)

Fig. 1 (A) Mir-124 was expressed in 3 kinds of esophageal carcinoma cells and human normal esophageal squamous epithelial cells

(B) It is observed that Mir-124 stated within 48 esophageal carcinoma tissues as well as adjacent normal tissues

Table 1 Correlation of appearance level of miR-124 with the clinicopathological features in EC patients

Characteristic	miR-124 expression		p-value
	Low(n=24)	High(n=24)	
Gender			0.507
Masculine	13	15	
Feminine	11	9	
Age(yr)			0.558
<50	6	7	
≥50	18	17	
Depth of invasion			0.016*
Mucosa	8	15	
Submucosa	16	9	
Differentiation			0.262
Good/Moderate	17	15	
Poor	7	9	
TNM staging			0.018*
I~II	6	14	
III~IV	18	10	
Lymph node metastasis			0.000*
Yes	15	7	
No	9	17	

p-value had been attained through Pearson chi-square assessment.

*Statistically significant (p<0.05).

3. Relationship between miR-124 expression and patient prognosis

We used Kaplan-Meier Survival Curve Investigation for the examination the impact miR-124 has at the prognosis of diseased persons with esophageal cancer. The results showed that within the period of existence of miR-124 high appearance patients had been considerably higher than that of low expression group. It is revealed through the table 2, the modification had been numerically noteworthy ($P < 0.05$). Study of Cox regression showed that miR-124 relative expression, stage TNM, extent of invasion and metastasis of the lymph node were independent risk factors for prognosis in patients with esophageal cancer, as shown in table 2.

Table 2-2 Multivariate investigation of numerous variables for RFS and OS

Variable	Univariate Cox's Regression analysis		Multivariate Cox's Regression analysis	
	Hazard ratio (95% CI)	p-value	Hazard ratio (95% CI)	p-value
miR-124(high vs. low)	2.621 (1.335-4.987)	0.007	1.540 (1.025-2.095)	0.032
Gender (masculine vs. feminine)	1.115 (0.732-1.444)	0.852	-	-
Age (<50 yr vs. ≥50 yr)	0.794 (0.472-1.316)	0.351	-	-
Depth of invasion	1.477 (1.035-2.068)	0.018	1.382 (1.056-2.075)	0.028
TNM staging(I~II vs. III~IV)	2.236 (0.975-3.408)	0.032	2.516 (1.108-3.204)	-
Differentiation(poor vs.good/moderate)	1.311 (1.032-2.107)	0.621	-	-
Lymph node metastasis(Yes vs. No)	1.502 (1.011-2.035)	0.027	1.426 (1.020-2.041)	0.012

p-value obtained through Cox relative dangers reversion.
RFS,rescurrence-free existence; OS ,general existence ;CI, assurance pause;

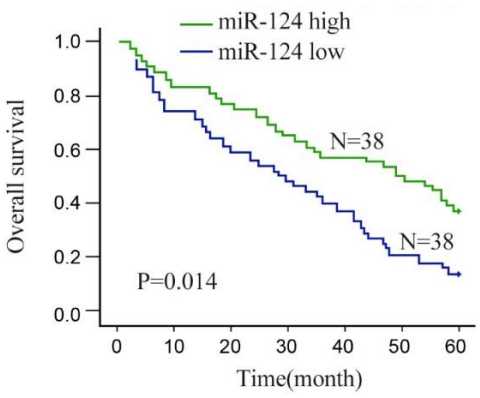


Fig.2 Correlation between miR-124 appearance phase and general subsistence

4. miR-124 inhibits cell proliferation of esophageal cancer cells

The oesophageal cancer cells were divided into the mimics group miR-124, the regulator set miR-124 mimics, the inhibitor set miR-124 and the control set

miR-124 inhibitor. RT-PCR observed appearance of miR-124 within the four groupings. The discoveries presented that within miR-124 mimics group the appearance level miR-124 had been significantly higher than within miR-124 mimics control set, though in miR-124 inhibitor group the appearance phase of miR-124 had been suggestively lesser than that of miR-124 inhibitor control group , suggesting positive transfection. CCK-8 findings displayed that miR-124 mimics cell propagation activity decreased significantly from the second day of transfection, while miR-124 inhibitor cell proliferation activity increased significantly, suggesting that miR-124 may obstruct cell propagation potential in oesophageal cancer cells, as shown in Figure 3.

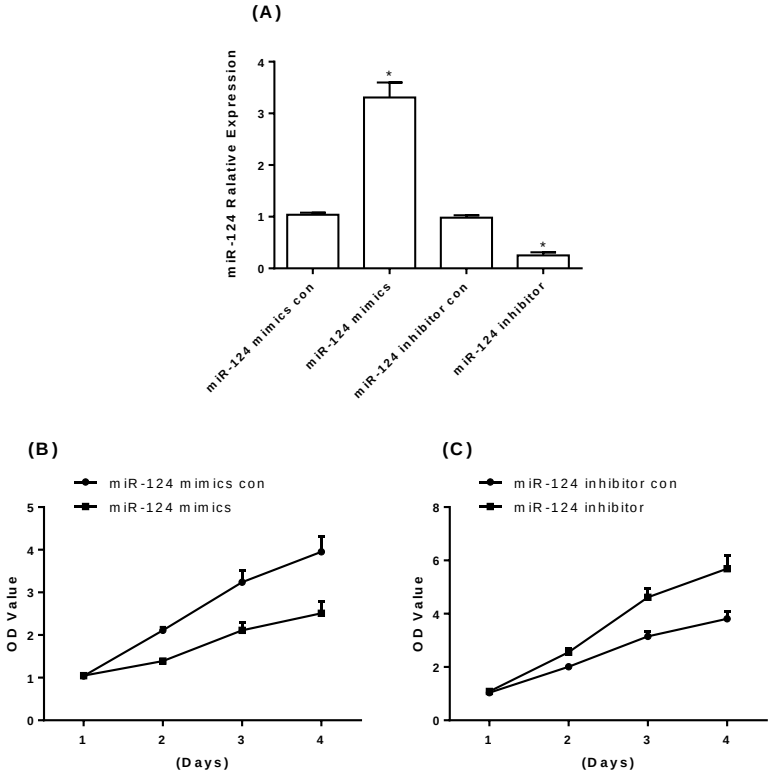
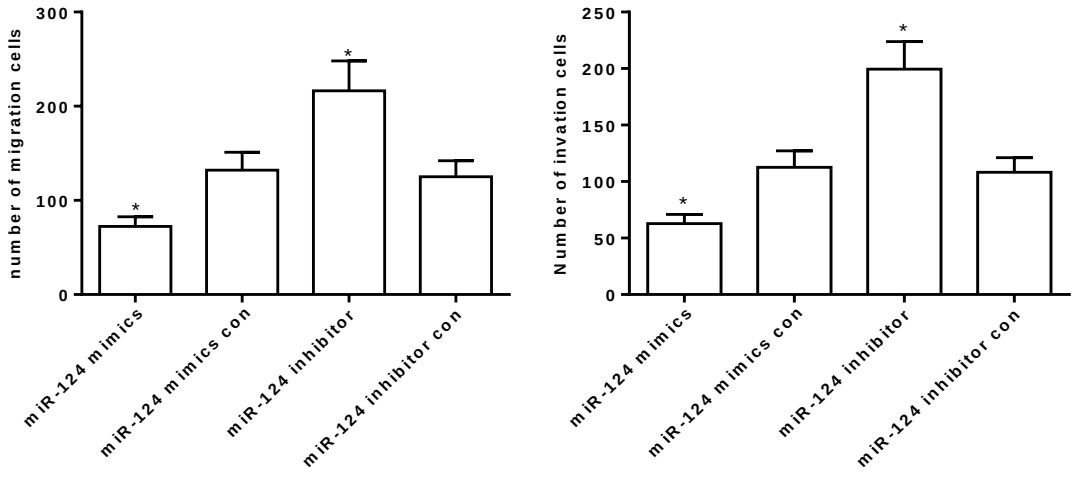


Fig.3 Effect of mir-124 on proliferation activity of esophageal carcinoma cells
(A) Mir-124 expression levels in cells of different transfection groups
(B) Proliferation activity of the three groups of cells

5. miR-124 inhibits cell migration and invasion of esophageal cancer cells

The outcomes of Transwell displayed that normal cell migration of miR-124 mimics set had been suggestively lesser as compared to miR-124 mimics regulator set, while the average cell number of miR-124 inhibitor group was significantly increased, indicating that miR-124 can inhibit cell migration in oesophageal cancer. Figure 4 (A, C) shows the migration of the cancer cells. Similarly, the average number of invasive cells within the miR-124 set of imitators was meaningfully reduced, while the average number of cells within the miR-124 set of inhibitors had been meaningfully increased, representing the miR-124 inhibits incursion of esophageal cancer cells, and it is revealed by the table Figure 4B & 4D.

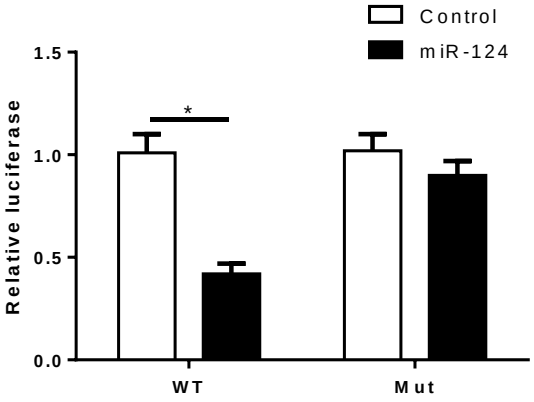


(C) (D)

Fig. 4 The number of cell migration and infiltration of two sorts of esophageal carcinoma cells after the overexpression of mir-124

6. miR-124 directly targets STAT3 expression

The TargetScan and miRanda target gene prediction websites were used to predict miR-124 downstream target genes, and the outcomes presented that miR-124 had a binding site with the STAT3 3'UTR end. The outcomes of double luciferase test displayed that miR-124 targets STAT3 directly, as shown in Figure 5.



(B)

Fig. 5 Double luciferase verified the direct targeting upshot of mir-124 at STAT3

7. Effect of miR-124 on STAT3 expression

RT-PCR has observed the STAT3 mRNA levels in different transfection groups. Outcomes exposed the phase of STAT3 mRNA in oesophageal cancer cells had decreased significantly after miR-124 overexpression, and modification had been numerically noteworthy (P<0.05). Western blotting analysis presented that after overexpression of miR-124, the appearance of STAT3 protein in oesophageal cancer cells reduced meaningfully, and the modification had been numerically noteworthy (P<0,05). MiR-124 has been shown to inhibit STAT3 expression as shown in Figure 6.

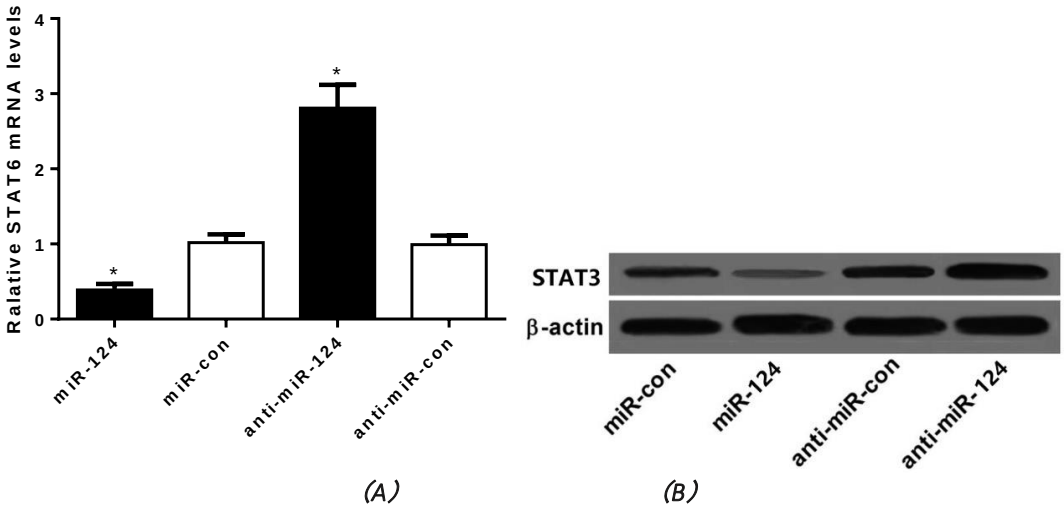


Fig. 6 Impact of mir-124 on STAT3 expression.
(A) Impact of mir-124 on STAT3 mRNA expression
(B) Impact of mir-124 on STAT3 protein expression

4. Discussion

Esophageal cancer is a tumor in the digestive tract that is highly malignant and involves multiple genes and factors. Differential forms of different miRNAs occur concerning progress and evolution of esophageal cancer. MiRNAs are also expressed differently for the esophageal squamous cell carcinoma and oesophageal adenocarcinoma. MiRNA is both highly potential and highly precise. According to Gao et al, miR-133a and miR-133b expression within the esophageal squamous cell carcinoma was significantly down-regulated and played a similar role as a tumor suppressor gene. The further analysis helps in revealing both miR-133a and miR-133b can target and inhibit their functions on FSCN1[5]. According to Cui et al, in esophageal adenocarcinoma, miR-194 and miR-200c had been significantly higher than within adjacent normal tissues, but not in oesophageal squamous cell carcinoma[6]. Winther et al . observed miR-21 expression by RT-PCR in 72 cases of oesophageal cancer tissue and adjacent normal tissue. Results displayed the miR-21 had been up-controlled within 63 cases of tissue of the esophageal cancer and the phase of appearance had been vital association

between tumor differentiation, metastasis of the lymph node and pathological grade[7].

MiR-124 is a miRNA discovered over the last few years. Its encoding gene is 17q23 in length and is 22 nucleotides. It includes the proliferation, division, and apoptosis of various cells within the human body. The study found miR-124 related the existence and growth of different cancers, especially gastrointestinal tumors[8~9]. It is argued that research works reveal that miR-124 can be stated differently in various cancers like hepatocellular carcinoma, colon cancer, gastric cancer, cholangiocarcinoma, lung cancer and breast cancer, along with others. By studying the manifestation reports of miRNAs within various tumor muscles and cells, based on tumors at different locations and miR-124 at different levels of expression, miR-124 can function not only as a tumor suppressor, but also as a proto-oncogene[10~12]. Cai et al . introduced miR-124 liposome-encapsulated into the liver cancer mice and found that miR-124 exerts a major anti-cancer effect by inhibiting the signaling cascade of IL-6 / STAT3 in hepatic cells [13]. Recently, the research work reveals that miR-124 inhibits in vitro

and in vivo growth of colorectal cancer cells by targeting main enzymes in the pentose phosphate pathway [14]. Several cancer-promoting molecules like the androgen receptor (AR) and EZH2 in prostate cancer cells have been identified in the prostate cancer research as direct downstream targets of miR-124[15]. Additionally, breast cancer studies have suggested that miR-124 can inhibit breast cancer cell production through targeting to reduce CDK4 representation [16].

Transcription Signal Transducer and Activator 3 (STAT3) possesses a connection of sign transduction and transcriptional activator family, widely

expressed in the cytoplasm and activated and transferred to center for attaching to DNA, involving signal transduction and transcription[17]. Due to its abnormally high expression in many human tumors and tumor propagation, diversity, apoptosis, angiogenesis, aggression and metastasis, and immune escape, STAT3 is an acute phase response factor (APRF) during interleukin 6, IL-6 signaling [18]. Pan found that the expression of EZH2 and other genes can be regulated by STAT3 to regulate the growth of gastric cancer cells[19]. Saini et al . reported up-regulation of STAT3 in ovarian cancer, which may be interested concerning ovarian tumor EMT by controlling the manifestation of E-cadherin [20]. Other studies have found that STAT3 in oesophageal cancer tissues and cells can be significantly up regulated and can induce EMT and enhance the movement and invasion of oesophageal cancer cells through the EMT transcription activator ZEB1[21].

The findings of this analysis demonstrated that miR-124 manifestation in the oesophageal cancer muscles and cell lines had been appreciably down-regulated. Among patients with esophageal cancer, miR-124 expression among tumor tissues was significantly associated with stage TNM, extent of invasion, and metastasis of the lymph nodes. Diseased persons with elevated miR-124 expression appear to have a longer life span. Results from cytology experiments showed that the cell propagation, immigration , and incursion of esophageal cancer cells diminished suggestively after cell transfection over-expression of miR-124, representing that miR-124 may constrain the propagation, immigration , and incursion of esophageal cancer cells and perform purpose as a tumor suppressor. Prediction in bioinformatics discovered that miR-124 possesses a requisite location to STAT3 's 3 'UTR and has been verified by dual luciferase assay. It is argued observed that miR-124 may immediately level STAT3, and has been further confirmed using RT-PCR and Western blotting, indicating that mi-124 can significantly decrease the expression of STAT3 mRNA and protein in oesophageal cells.

Briefly, it seems imperative that miR-124 is substantially low in oesophageal cancer expression, and their level of expression affects the patients' degree of disease progression and prognosis. MiR-124 can prevent the increase, resettlement, and aggression of oesophageal cancer cells, and plays a similar act like a tumor suppressor. The potential mechanism is to target STAT3. MiR-124 may have wide potential in relevance concerning the diagnosis, medication and prognosis of esophageal cancer, depending on the work focused on miR-124 transcriptional control mechanism, target genes and their target gene mechanism

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