Exosomes miR-184 and miR-3913-5p are involved in Osimertinib resistance in non-small cell lung cancer patients with exon 21 L858R mutation

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Research

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Abstract

Background

The third-generation of EGFR-TKI Osimertinib has become an important treatment option for patients with EGFR-mutant advanced NSCLC. In recent years, more and more studies have begun to pay attention to the ability of miRNAs in exosomes secreted by tumor cells to transmit resistance information. The mechanisms of exosomal miRNAs involved in Osimertinib resistance need to be studied.

Methods

We constructed an Osimertinib-resistant cell line H1975-OR, and collected the exosomes from the cells of the drug-resistant strain and the sensitive strain, and extracted respective RNA for sequencing. We also compared miRNAs in serum exosomes of three patients before and after resistance to Osimertinib. Enlarged samples were then validated in 64 NSCLC patients.

Results

Cluster analysis of target genes revealed that miRNAs in exosomes participate in Osimertinib resistance mechanisms through the activation of bypass pathways (RAS-MAPK pathway abnormality, PI3K pathway activation). MiR-184 and miR-3913-5p increased significantly in serum exosomes of patients with later Osimertinib resistance. These two miRNAs mainly cause EGFR resistance in two types of NSCLC patients, EGFR exon 21 L858R mutation and T790M positive.

Conclusions

Exosomes miR-184 and miR-3913-5p mainly cause Osimertinib resistance in lung cancer patients containing EGFR exon 21 L858R+ and T790M+. They are regarded as important biomarkers of third-generation EGFR-TKI resistance. This not only enriches the application of liquid biopsy in lung cancer drug resistance, but also provides a direction for future targeted drug research.

Background

Epidermal growth factor receptor (EGFR) mutations are the most common genetic mutations in non-small cell lung cancer (NSCLC)[1]. The PIONEER study (NCT01185314)[2] showed that the EGFR mutation rate in Chinese population reached 50.2%. Among them, exon 19 deletion (48.9%) and exon 21 L858R point mutation (45.4%) were more common[2, 3]. 50–60% NSCLC patients with EGFR mutations usually develop acquired resistance to TKI after first- and second-generation EGFR-TKI therapy[4], of which, p.Thr790Met (T790M) point mutation in exon 20 is the most common. The FLAURA study, which received wide attention at the 2019 European Society for Medical Oncology(ESMO) congress, confirmed that
EGFR-mutant NSCLC patients treated with the third-generation EGFR-TKI Osimertinib could extended 6.8 months of overall survival (OS) compared to patients receiving Gefitinib or Erlotinib therapy.

However, patients treated with Osimertinib will inevitably develop resistance.

Exosomes (extracellular vesicles at 30–150 nm) transmit biological information and participate in cell mediated biological activities by releasing large amounts of proteins, RNA, lipids and other biomolecules to the extracellular environment. MicroRNAs (miRNAs) in exosomes are not digested by RNases, which can be transported between cells and participate in epigenetics. Existing literature has shown that exosomes miRNAs secreted by tumor cells play an important role in drug resistance. It remains to be seen whether exosomes are involved in resistance to the third-generation EGFR-TKI Osimertinib.

In this study, we will elaborate on whether miRNAs in exosomes, especially before and after resistance to Osimertinib, transmit relevant information between tumor cells, so as to change the sensitivity of patients. By collecting exosomes from the supernatant of drug-resistant cells and sensitive cells of Osimertinib, comparing plasma exosomes of matched patients, the next-generation RNA-seq test was performed to find key miRNAs. Later, we verified the role of key miRNAs in Osimertinib resistance in larger population samples, in an attempt to elucidate the mechanism of exosomal miRNAs involved in Osimertinib resistance. We hope to provide directions for the treatment of NSCLC patients with Osimertinib resistance.

**Materials And Methods**

**Cell culture and establishment of resistant strains**

The lung adenocarcinoma cell line H1975 was purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Osimertinib was obtained from Selleck Chemicals (Houston, TX, USA). H1975 cells were exposed to Osimertinib at 0.05 µM for about 72 hours, and then the cells were transferred to drug-free medium and screened for drug-resistant cell lines. After the above steps, the drug concentration increased from 0.05 µM to 5 µM. Six months later, the H1975 cells became the resistant strain of Osimertinib, H1975-OR. All cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and antibiotics (100 units /ml penicillin and 100 µg/ml streptomycin). All cells were incubated in 37 °C, 5% CO2 humidified air.

Cell proliferation was measured in three replicates by the MTT method. A non-linear regression function was used to fit the dose-response curve in Graphpad Prism 5.0. After the IC50 values of the semi-inhibition rates of the two cells were obtained, the drug resistance index was calculated. Both MTT and dimethyl sulfoxide (DMSO) are from Sigma, St. Louis, Missouri, USA. RPMI 1640 medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco Life Technologies (Grand Island, NY, USA).

**Patient selection and sample collection**
A total of 67 NSCLC patients were included in this study in the Department of Respiratory and Critical Care Medicine at Jinling Hospital from December 2018 to October 2019. Three pair of blood samples were collected before and after resistance to Osimertinib. Another 37 patients were resistant to Gefitinib but have not yet developed Osimertinib resistance, and 27 had Osimertinib resistant. In addition, 10 healthy people were recruited from the physical examination center of Jinling Hospital. All participants signed informed consent. This study was approved by the ethics committee of Jinling Hospital.

**Exosomes isolation**

In order to isolate the effect of serum, when the cells grew to 80% full in a petri dish, H1975 and H1975-OR cells were cultured in complete RPMI-1640 medium without added serum. After 24 hours, the cell culture supernatant was collected and exosomes were separated by differential ultracentrifugation as described in[13]. To remove cell debris, the supernatant was centrifuged at 300 × g for 5 min and then at 3000 × g for 10 min. The supernatant was then centrifuged at 10,000 × g for 30 min. The supernatant was passed through a filter with an aperture of 0.22 µm (Millipore, USA). The filtered supernatant was transferred to a clean ultracentrifuge tube and ultracentrifuged at 4 °C, 120000 × g for 70 min. After discarding the supernatant, the pellet was resuspended in an appropriate amount of sterile phosphate buffered saline (1 × PBS). Extracted exosomes can be used for immediate downstream experiments or stored in a -80 °C refrigerator.

Serum samples were extracted using a commercial kit Total Exosome Isolation (from serum) (invitrogen, 4478360). The required volume of clear serum was transferred to a new test tube and 0.2 volume of total exosomal separation (from serum) reagent was added. Mix the serum/ reagent mixture, whether through eddy current or pipe up and down, to make the solution turbid. After incubation at 4 °C for 30 min, centrifuge at 10,000 × g for 10 min at room temperature. Aspirate and discard the supernatant. Exosomes are contained in granules at the bottom of the test tube. Exosomes were resuspended using half the volume of the initial serum volume of sterile phosphate buffered saline (1 × PBS).

**Transmission electron microscopy, size distribution analysis and Western blot**

Exosome morphology was observed by transmission electron microscopy. The exosome suspension was mixed with an equal volume of 4% paraformaldehyde, and 10 µl of the mixture was placed on a clean copper grid (RT) at room temperature. Uranyl acetate staining was negative. The images were acquired by observation with a JEOL 1200EX TEMSCAN microscope. Exosomal suspensions were analyzed for particle size by dynamic light scattering (DLS) (Nanosizer ™ instrument, Malvern Instruments, UK).

The extracted exosomes were resuspended in cell lysate (Beyotime, Nantong, China) added with 1% PMSF. The protein concentration of exosomes was determined by Pierce BCA protein detection kit (Thermo Fisher Scientific, Rockford, IL). The twelve alkyl sulfate polyacrylamide gel electrophoresis was prepared with 20 µg protein on each sample. Anti-CD63, anti-TSG101, anti-β-actin, and anti-GAPDH were purchased from (Abcam, Cambridge, UK).
**Library construction and sequencing**

The total RNA in exosomes was extracted by using TRizol reagent (Invitrogen, Carlsbad, CA) according to the instructions. The NanoPhotometer spectrophotometer was used to detect RNA purity (OD260 / 280 and OD260 / 230 ratios). After using the Qubit2.0 Fluorometer to accurately quantify the RNA concentration, the Agilent 2100 bioanalyzer was used to accurately detect the integrity of the RNA. The total RNA of exosomes was reverse transcribed into cDNA. The starting RNA of the library was total RNA, and the total amount was ≥ 1 µg.

The small RNA library construction process is mainly summarized as follows: starting from the quality-tested RNA, 3’ end adaptor ligation, reverse transcription primer hybridization, 5’ end adaptor ligation, reverse transcription into DNA, and then PCR amplification. After the library construction is completed, first use Qubit2.0 for preliminary quantification, adjust the library to 1 ng/µl, and then use the Agilent 2100 to detect the length of the library insert. After meeting the expectations, use the Q-PCR method to determine the effective concentration of the library. Accurate quantification (effective library concentration > 2 nM) to ensure library quality.

After the library was qualified, different libraries were pooled according to the requirements of effective concentration and target offline data volume for illumine sequencing. The basic principle of sequencing was sequencing by synthesis. Added four fluorescently labeled dNTPs, DNA polymerases, and adaptor primers to the sequencing flow cell for amplification. When each sequencing cluster extended the complementary strand, each fluorescently labeled dNTP could release the corresponding fluorescence. The sequencer obtained the sequence information of the fragments to be detected by capturing the fluorescent signal and converting the optical signal into a sequencing peak by computer software.

**Sequence read analysis**

The raw data from small RNA-seq includes linker sequences and sequencing low-quality sequences. In order to ensure the accuracy of information analysis, the sequencing raw data needs to be filtered to obtain clean data, and subsequent bioinformatics analysis is performed based on clean data. The Qphred score \( Q_{phred} = 10\log_{10} (e) \) is used to represent the base quality value (Quality Score) to measure the quality of each base in the sequencing reads. Since miRNA is a small RNA with a length of about 22nt, the length of the sequence fragment mainly distributed around 22nt is selected as Clean reads data. The miRDeep2 software was used to analyze the miRNA expression abundance. According to the mapping position of read on the genome, it was necessary to verify whether the position of read and the known miRNA mature sequence were consistent. Cluster analysis and correlation analysis between samples (Pearson's Correlation Coefficient) were performed on the miRNA family in each sample. All sequencing raw data has been uploaded to the SRI database. [SubmissionID: SUB7187450; BioProject ID: PRJNA615931] (http://www.ncbi.nlm.nih.gov/bioproject/615931)

**Target gene analysis**
The analysis was performed by DESeq2 (no biological duplicate samples use DESeq or edgeR), and miRNAs with \[\text{logFoldChage} > 1\] and \(p\) value \(< 0.05\) were selected as miRNAs with significant differences. The volcano map of the results of miRNA differential analysis and the clustered heat map of miRNA expression of the samples were drawn. TargetScan was used to calculate a weighted context + + score to predict the target genes of different known miRNAs. These target gene functions were classified through a database established by the Gene Ontology Consortium, and GO enrichment analysis was completed. At the same time, the first 10 target genes of each sample were selected for KEGG Pathway enrichment analysis according to the parameters, so as to identify the downstream molecular metabolic pathways of these target genes.

### Quantitative reverse transcription PCR

As aforementioned, exosomes were extracted from the patient's serum, and then the total RNA of the exosomes was extracted by TRizol reagent. RNA was quantified and evaluated using NanoDrop® ND-2000 (Thermo Fisher Scientific, USA). We used the miRNA first-strand cDNA synthesis (tailing method) kit from Shanghai Sangon Biotech, using the method of Poly (A) tailing reaction and cDNA synthesis reaction simultaneously. According to the instructions of the experiment, we obtained all cDNA products of miRNA then Real Time PCR experiments were performed. In RT-PCR, the universal downstream primer Universal PCR Primer R and the endogenous reference U6 (Universal U6 Primer F) in the above kit are used uniformly. The upstream primers of several miRNAs are: miR-184: 5'-catGGACGGAGAActGAtAAGGGt-3'; miR-3913-5p: 5'-acggTTTGGGACTGATCTTGATGTCT-3'; miR-4746-5p: 5'-CCGGTCCCAGGAGAACC-3'; miR-3614-5p: 5'-CCACTTGGATCTGAAGGCTGC-3'. RT-PCR was performed on an ABI 7500 real-time polymerase chain reaction (PCR) system (Applied Biosystems, Foster City, CA). The levels of all miRNAs were determined using the 2-\(\Delta\Delta CT\) method: \(\Delta CT\) (target) = CT (target) − CT (U6).

All the above experiments were repeated twice.

### Statistical analysis

The above data were mainly analyzed and mapped using SPSS 22.0 system (SPSS, Inc. Chicago, IL) and Graphpad Prism5. The differences in miRNAs levels between the two groups were mainly determined by the Mann-Whitney rank sum test for non-parametric data. The correlation between Exo-miR-184 and Exo-miR-3913-5p with clinicopathological features was determined by \(\chi^2\) test. ROC curves were also used to determine diagnostic value. A \(P\) value less than 0.05 indicates a statistical difference.

### Results

#### Construction of Osimertinib-resistant strains and isolation of exosomes

In the commonly used lung cancer cell lines, H1975 carries EGFR-L858R-sensitive mutation or EGFR-T790M drug-resistant mutation. Therefore, we decided to use H1975 to construct a drug-resistant cell line for the study of Osimertinib resistance. The drug-resistant strain H1975-OR was established by increasing
the concentration of Osimertinib. Six months later, MTT method was used to determine the cell viability of the sensitive strain H1975 and the resistant strain H1975-OR (Fig. 1A). The average IC50 values of these two cells were 4636 nM and 12101 nM, respectively, with significant differences (p = 0.0215) (Fig. 1B). RI was 2.61.

The circular vesicle-like exosomes were obtained from different cell supernatants by ultracentrifugation. After TEM identification (Fig. 1C) and DLS analysis (Fig. 1D), the average particle size was 97.79 nm. Meanwhile, the marker proteins CD63 and TSG101 of exosomes were identified by Western blot (Fig. 1E).

**Screening for differential exosome miRNAs in H1975-OR and H1975 cell supernatant**

Exosomes were extracted and sequenced from the supernatants of H1975 and H1975-OR cells. The RNA-Seq data were analyzed by using the software of DESeq2 (no biological duplicate samples using DESeq or edgeR) and the corresponding FDR (p value corrected FDR value, the smaller the FDR value, the more significant the difference) with log2 (FC) (multiple change in expression level of the experimental group relative to the control group). The results showed that there were 1138 differential exosomal miRNAs in the two groups. [log2FoldChage] > 1 and p value < 0.05 are the identification criteria for significantly different miRNAs. In order to see the differences more intuitively, the volcano map (Fig. 2A) of the miRNA difference analysis and the clustered heat map (Fig. 2B) of the top 50 miRNA expression levels were drawn respectively. The expression levels of exosomes miR-6087, miR-99b-5p, and miR-7641 in H1975-OR supernatant were significantly higher than those in H1975 supernatant exosomes. MiR-378a-3p, miR-25 -5p, miR-1293, etc. were significantly reduced (Fig. 2C).

**Functional enrichment analysis of differentially expressed miRNA target genes in exosomes**

In order to further understand the biological functions of the exosomes of Osimertinib-resistant cells, the target genes of differential miRNAs were classified through the GO (Gene Ontology) database (Fig. 2D), including biological process, molecular function and cell component. According to the selected target genes of different miRNAs, the first 10 target genes of each miRNA are selected, and the hypergeometric distribution relationship between these target genes and a specific branch (or branches) in go classification is calculated. GO analysis would return a p-value for each miRNA predicted target gene. A small p-value indicates that the target genes of differential miRNAs are enriched in this GO. Most genes involved in cell components, such as "extractor exosome", "extractor region" and "extractor space", once again verified that miRNAs are derived from exosomes. "Transcription, DNA templated", "positive regulation of gene expression" and other gene aggregations related to biological pathways also indicate that the miRNA of exosomes plays an important role in the resistance of Osimertinib (Table 1).
Table 1
GO terms enriched for miRNAs target genes up-regulated in H1975-OR.

| GO ontology          | GO accession | GO terms                      | Number of genes | P-Value |
|----------------------|--------------|--------------------------------|-----------------|---------|
| Cellular Component  | GO:0005576   | extracellular region           | 70              | 0.00145 |
|                      | GO:0005615   | extracellular space            | 43              | 0.00518 |
|                      | GO:0005886   | plasma membrane                | 72              | 0.00553 |
|                      | GO:0005737   | cytoplasm                      | 142             | 0.00579 |
|                      | GO:0031012   | extracellular matrix           | 12              | 0.0207  |
|                      | GO:0070062   | extracellular exosome          | 68              | 0.0388  |
|                      | GO:0016021   | integral component of membrane | 95              | 0.0152  |
| Biological Process   | GO:0071356   | cellular response to tumor necrosis factor | 7 | 0.00897 |
|                      | GO:0006351   | transcription, DNA-templated   | 59              | 0.0309  |
|                      | GO:0010628   | positive regulation of gene expression | 12 | 0.0368  |
|                      | GO:0045087   | innate immune response         | 18              | 0.0438  |
| Molecular Function   | GO:0003714   | transcription corepressor activity | 11 | 0.00122 |
|                      | GO:0000166   | nucleotide binding             | 28              | 0.0437  |

KEGG pathway enrichment analysis can help us understand the molecular metabolic pathway information or complex biological activities involved in differential genes. According to the parameters, the first 10 target genes of different miRNAs were selected for pathway enrichment (Fig. 2E). "Metabolic pathways" accounted for the largest proportion among the differential miRNA target genes, followed by "PI3K-Akt signaling pathway", "Ras signaling pathway", "Cytokine-cytokine receptor interaction", "Non-small cell lung cancer", etc. These are common pathways closely related to EGFR-TKI in NSCLC. Previous studies have shown that EGFR-independent mechanisms of Osimertinib resistance include bypass activation of PI3K pathway, which can be achieved through PIK3CA mutation/amplification and PTEN deletion. The sequencing results in the exosomes of H1975-OR cell supernatants confirmed that exosome PI3K/Akt pathway activation was involved in Osimertinib resistance.

In the dot plot (Fig. 2F), the most significant pathway enriched in Pathway analysis of known differential miRNA target genes is the Ras signaling pathway, and the Pathway analysis of novel differential miRNA target genes is concentrated in the MAPK signaling pathway (Supplementary Fig. 1). It is well known that RAS-MAPK pathway aberrations can cause EGFR-mutant NSCLC patients to develop resistance to Osimertinib. This also shows that exosomes participate in Osimertinib resistance mainly by affecting activation of the RAS-MAPK pathway.
Differential miRNAs in serum exosomes before and after Osimertinib resistance

A 62-year-old male NSCLC patient (Patient 2, Treat3 & Control3) was treated with Gefitinib after molecular testing that revealed EGFR 19 deletion mutation. One year later, he started oral AZD9291 due to new brain and bone metastases. The cancer progressed after 19 months later. Blood samples were collected from the patient at the start of Osimertinib treatment and resistance. Similarly, blood samples were collected from another 53-year-old female patient (left lung adenocarcinoma stage IVB, EGFR 21 L858R mutation) (Patient 1, Treat2 & Control2) and a 58-year-old female patient (left lung adenocarcinoma stage IVB, EGFR 19 deletion) (Patient 3, Treat4 & Control4) before and after resistance to Osimertinib. Exosomes were extracted from the serum using exosome extraction kit, and RNA was extracted for sequencing. Exosomes were also identified by TEM, DLS analysis and Western Blot (Fig. 3A-C).

Based on exosome transcriptome data obtained from two RNA-seqs, Pearson's Correlation Coefficient (R2) was used to assess the correlation between the cell supernatant and different samples from the patient's serum, and the Correlation heat map matrix was drawn (Fig. 3D). The miRNAs in serum exosomes were compared before and after drug resistance in three patients. The miRNAs with significantly different expression were hierarchical clustered, and the clustering heat map was drawn (Fig. 3E). The first patient listed in the figure had EGFR 21 L858R mutation, except for a large number of new differential miRNAs. Cluster analysis showed that miR-206, miR-200b-3p and miR-514b-5p were significantly up-regulated in patients with drug resistance to Osimertinib. However, the other two patients with EGFR 19 depletion significantly upregulated more miRNAs, including miR-25-5p, miR-184, miR-3913-5p and so on.

**Increased miR-184 and miR-3913-5p in exosomes after Osimertinib resistant**

We performed the overlapping analysis of differential miRNAs obtained by sequencing exosomes in the cell supernatant (treat1) and three patients (treat2-4) by way of Wayne map (Fig. 4A, B). Among the exosome miRNAs up-regulated in the Osimertinib-resistant group compared with the sensitive group, no common differential miRNAs that overlapped in the four groups were found (Fig. 4A). However, in pairwise comparison, it was found that miR-184 overlapped in treat1 and treat4, miR-3913-5p was shared in treat3 and treat4, and miR-3614 co-occurred in treat1 and treat2 (Fig. 4A). These three miRNAs can be further studied. Similarly, miR-3614-5p, miR-4746-5p, miR-378i are the focus of miRNA in the down-regulation group (Fig. 4B).

In order to verify several significantly up-regulated Exo-miRNAs in the RNA-seq results, we decided to expand the sample size for experiments. Serum samples from 37 NSCLC patients with Gefitinib-resistance and 27 patients with Osimertinib-resistance were collected as controls (GR) and experimental groups (OR) before and after Osimertinib resistance. QPCR verified that exosome miR-184 in the serum of drug-resistant patients was significantly higher (p = 0.0325) (Fig. 4C), and exosome miR-3913-5p was
significantly increased in the Osimertinib resistant group ($p = 0.0169$) (Fig. 4D), which was consistent with the previous sequencing results. Combining these two exosome miRNAs, it was found that these two miRNAs in the serum exosomes of NSCLC patients were significantly up-regulated after Osimertinib resistance ($p = 0.0092$) (Fig. 4E, F).

The relationship between exosome miRNA levels and the clinicopathological characteristics of patients (including age, gender, smoking history, TNM stage, primary tumor size, platelet count (PLT), lactate dehydrogenase (LDH), carcinoembryonic antigen (CEA), distant metastasis, etc.) is summarized in Table 2&3. Exosome miR-184 was correlated with LDH levels ($p = 0.018$) (Table 2). Exosome miR-3913-5p was associated with TNM stage ($p = 0.045$), PLT ($p = 0.024$), tumor marker CEA ($p = 0.045$), distant metastases ($p = 0.049$), especially bone metastases ($p = 0.03$) (Table 3). However, there was no significant correlation between miRNA levels of the two exosomes and age, gender, smoking status, ECOG score, and primary tumor size.
### Table 2
Correlation between serum exosomal miR-184 levels in patients with NSCLC and clinicopathological characteristics.

| Clinicopathological parameters | Number of patients(%) | Low | High | P     |
|-------------------------------|-----------------------|-----|------|-------|
| Exosomal miR-184              |                       |     |      |       |
| Age(years), Median            |                       |     |      | 0.777 |
| ≥60                           | 33                    | 15  | 18   |       |
| ≥ 60                          | 31                    | 13  | 18   |       |
| Gender                        |                       |     |      | 0.899 |
| Male                          | 28                    | 12  | 16   |       |
| Female                        | 36                    | 16  | 20   |       |
| Smoking history               |                       |     |      | 0.353 |
| Never smoking                 | 49                    | 23  | 26   |       |
| Now/once smoking              | 15                    | 5   | 10   |       |
| ECOG Score                    |                       |     |      | 0.226 |
| 0–1                           | 53                    | 25  | 28   |       |
| ≥ 2                           | 11                    | 3   | 8    |       |
| Primary tumor                 |                       |     |      | 0.705 |
| ≤ 4 cm                        | 19                    | 9   | 10   |       |
| ≥ 4 cm                        | 45                    | 19  | 26   |       |
| TNM                           |                       |     |      | 0.317 |
| I-IVA                         | 25                    | 9   | 16   |       |
| IVB                           | 39                    | 19  | 20   |       |
| PLT                           |                       |     |      | 0.411 |
| Low(≤ 146)                    | 13                    | 7   | 6    |       |
| High(>146)                    | 51                    | 21  | 30   |       |
| LDH                           |                       |     |      | 0.018*|
| Low(≤ 199)                    | 29                    | 8   | 21   |       |
| High(>199)                    | 35                    | 20  | 15   |       |

(* p < 0.05)
|                      | Exosomal miR-184 |
|----------------------|------------------|
|                      |                  |
| CEA                  | 0.129            |
| Low (≤ 8.605)        | 25 8 17          |
| High (>8.605)        | 39 20 19         |
| Distant metastasis   |                  |
| No                   | 17 5 12          |
| Yes                  | 47 23 24         |
| Bone metastasis      |                  |
| No                   | 23 9 14          |
| Yes                  | 41 19 22         |
| Brain metastasis     |                  |
| No                   | 36 14 22         |
| Yes                  | 28 14 14         |

(* p < 0.05)
Table 3
Correlation between serum exosomal miR-3913-5p levels in patients with NSCLC and clinicopathological characteristics.

| Clinicopathological parameters | Number of patients(%) | Low | High | P       |
|-------------------------------|------------------------|-----|------|---------|
| **Exosomal miR-3913-5p**      |                        |     |      |         |
| Age(years), Median            |                        |     |      | 0.714   |
| <60                           | 32                     | 25  | 7    |         |
| ≥ 60                          | 31                     | 23  | 8    |         |
| Gender                        |                        |     |      | 0.427   |
| Male                          | 28                     | 20  | 8    |         |
| Female                        | 35                     | 28  | 7    |         |
| Smoking history               |                        |     |      | 0.321   |
| Never smoking                 | 48                     | 38  | 10   |         |
| Now/once smoking              | 15                     | 10  | 5    |         |
| ECOG Score                    |                        |     |      | 0.064   |
| 0–1                           | 52                     | 42  | 10   |         |
| ≥2                            | 11                     | 6   | 5    |         |
| Primary tumor                 |                        |     |      | 0.64    |
| ≤ 4 cm                        | 18                     | 13  | 5    |         |
| > 4 cm                        | 45                     | 35  | 10   |         |
| TNM                           |                        |     |      | 0.045*  |
| I-IIVA                        | 24                     | 15  | 9    |         |
| IVB                           | 39                     | 33  | 6    |         |
| PLT                           |                        |     |      | 0.024*  |
| Low(≤ 146)                    | 13                     | 13  | 0    |         |
| High(>146)                    | 50                     | 35  | 15   |         |
| LDH                           |                        |     |      | 0.427   |
| Low(≤ 199)                    | 28                     | 20  | 8    |         |
| High(>199)                    | 35                     | 28  | 7    |         |

(* p < 0.05)
|                          | Exosomal miR-3913-5p | CEA     |
|--------------------------|----------------------|---------|
|                          |                      | **0.045*** |
| Low (≤ 8.605)            | 24                   | 15      |
| High (> 8.605)           | 39                   | 33      |
| **Distant metastasis**   | **0.049***           |         |
| No                       | 17                   | 10      |
| Yes                      | 46                   | 38      |
| **Bone metastasis**      | **0.03***            |         |
| No                       | 23                   | 14      |
| Yes                      | 40                   | 34      |
| **Brain metastasis**     | 0.112                |         |
| No                       | 35                   | 24      |
| Yes                      | 28                   | 24      |

(* p < 0.05)

**Exosomal miRNAs induce resistance to Osimertinib in patients with EGFR 21 L858R mutations and T790M+**

Of the 64 NSCLC patients, 28 were patients with EGFR exon 19 deletion and 36 had mutations in the exon of EGFR 21. In all patients with exon 19 deletion, there was no significant difference in serum exosome miR-184 (p = 0.776) and miR-3913-5p (p = 0.631) between the two groups before and after drug resistance (Fig. 5A, B). However, in all patients with mutations in L858R in exon 21 of EGFR, serum exosomes miR-184 was significantly elevated in Osimertinib-resistant patients (p = 0.0104) (Fig. 5C). Exo-miR-3913-5p (p = 0.0085) was also significantly involved in patients with Osimertinib-resistance (Fig. 5D). ROC curve analysis showed that the AUC of exosome miR-184 was 0.736, while the AUC of exosome miR-3913-5p was 0.759 (Fig. 5E, F), indicating that these two miRNAs played important roles in Osimertinib-resistance and were mainly involved in drug resistance of patients with EGFR 21 L858R. Because the sensitive group (GR) was selected for Gefitinib-resistant patients, the criteria for clinical resistance in these patients were enlarged lesions or new organ metastases. After re-sequencing, most patients (n = 44) showed T790M-positive mutations in EGFR-TKI resistance, but a few patients (n = 20) did not detect T790M-positive ([Supplementary Fig. 2](#)). In T790M + Osimertinib-resistant patients, the level of exosome miR-3913-5p was significantly increased (p = 0.013) (Fig. 5H), and exosome miR-184 resistant group was also higher than the sensitive group, but there was no significant difference (p = 0.065) (Fig. 5G).

**Discussion**

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Osimertinib (AZD9291) is the first third-generation EGFR-TKI to be approved by the FDA and EMA for the treatment of NSCLC[5]. It is selective for EGFR-TKI sensitization and T790M resistance mutations, and has less effect on WT-EGFR[14]. Regardless of first-line or second-line use of Osimertinib, similar to other EGFR-TKIs, patients still inevitably develop resistance after receiving Osimertinib, which greatly limits the long-term clinical benefits of this targeted drug[15]. The mechanism of Osimertinib resistance depends on the high tumor heterogeneity of NSCLC, which is divided into two aspects: EGFR-dependent and EGFR-independent[16]. Moreover, previous studies have shown that the mechanism of resistance after first-line or second-line use of Osimertinib varies with clonal evolution[17]. Our study focused on patients with advanced NSCLC who used non-first-line Osimertinib. Patients enrolled in the sensitive group (GR) were those who were sensitive to Osimertinib after Gefitinib resistance, while patients in the resistant group (OR) were those who had developed clinical resistance to Osimertinib. This could ensure the homogeneity of these two groups of samples.

The most common drug-resistance mechanism that dependent on EGFR mutations is C797S mutation occurring on exon 20, which has previously been reported to account for 10–26% of second-line Osimertinib resistance[18]. G796 mutation adjacent to C797S, in addition, there are multiple mutation sites such as L792, L718, G719, G724 and EGF overexpression[19]. However, in our study, of the 27 patients with Osimertinib resistance, only 3 (11.11%) had the C797S mutation confirmed by molecular testing. Of course, there were also a large number of patients who did not perform genetic testing again due to economic reasons.

Among the mechanisms of drug resistance independent of EGFR, mainly are activation of bypass signals, abnormalities in downstream pathways, and histological transformation. The most common in activated bypass pathways are MET amplification[20] and HER2 amplification[21]. Preclinical studies have shown that Osimertinib combined with Crizotinib, a c-MET inhibitor, can overcome MET amplification after Osimertinib resistance[22]. The abnormality of RAS-MAPK pathway is an important mechanism of Osimertinib resistance. Ortiz-Cuaran et al. [23] confirmed that when acquired resistance to second-line Osimertinib was developed, re-biopsy of the tumor revealed a KRAS G12S mutation. Kim et al. [24] reported a case of MAPK1 mRNA overexpression in a patient who received second-line treatment with Osimertinib in advanced stages. This is consistent with our sequencing results of exosomes. In Fig. 2F, the most obvious pathway enriched by the known differential miRNA target gene of exosomes in the supernatants of drug-resistant (H1975-OR) and sensitive (H1975) strains is Ras signaling pathway. As shown in supplementary Fig. 1, MAPK signaling pathway is the most abundant one among the novel differential miRNA target genes with the largest number of genes involved. This indicates that the mechanism of exosomal miRNA involvement in Osimertinib resistance mainly affects the abnormality of the RAS-MAPK pathway. In addition, the PI3K pathway has a place in bypass activation. It is currently believed that PIK3CA mutation or amplification and PTEN deletion can lead to PI3K pathway activation[24]. E545K, E542K, R88Q, N345K, and E418K mutations were related to second-line Osimertinib resistance. E545K mutation was the most common and was verified in vitro[25]. In our study, the PI3K-Akt signaling pathway was found in both the cell supernatant and the exosome miRNA target gene enrichment pathway in patient serum. Exosomal miRNA can convey Osimertinib resistance information
to affect PI3K pathway activation. Osimertinib resistance is also related to changes in cell cycle genes, including cyclin D1, cyclin D2, cyclin E1, cyclin-dependent kinase (CDK) 4 and CDK6[26]. In the sequencing results, exosome miR-6087 increased significantly in the drug-resistant group. Its target gene was CCND1 (Table 4), which encodes the cyclin D1 protein. This again demonstrates that exosomes participate in Osimertinib resistance by causing bypass activation.
Table 4
The mechanism by which exosomal miRNAs participate in Osimertinib resistance is bypass pathways activation.

| Term                        | GeneName | miRNA                          | log2FoldChange | stat |
|-----------------------------|----------|--------------------------------|----------------|------|
| Ras signaling pathway       | PDGFC    | hsa-let-7d-3p(hsa-let-7d)      | 0.020946       | up   |
|                             | NGF      | hsa-let-7e-5p(hsa-let-7e)      | 4.10E-08       | up   |
|                             | FGF7     | hsa-miR-30e-5p(hsa-mir-30e)    | 0.012066       | up   |
|                             | FGF7     | hsa-miR-30c-5p(hsa-mir-30c-2)  | 0.026564       | up   |
|                             | FGF7     | hsa-miR-30c-5p(hsa-mir-30c-1)  | 0.025462       | up   |
|                             | PIK3CD   | hsa-miR-7704(hsa-mir-7704)     | 0.000227       | up   |
|                             | GNGT2    | hsa-miR-1246(hsa-mir-1246)     | 3.44E-06       | up   |
| Ras signaling pathway       | BAD      | hsa-miR-1292-5p(hsa-mir-1292)  | 0.009353       | down|
|                             | RASA3    | hsa-miR-95-3p(hsa-mir-95)      | 0.003583       | down|
|                             | PLA2G2A  | hsa-miR-9-5p(hsa-mir-9-3)      | 0.000403       | down|
|                             | PLA2G2A  | hsa-miR-9-5p(hsa-mir-9-2)      | 0.000403       | down|
|                             | PLA2G2A  | hsa-miR-9-5p(hsa-mir-9-1)      | 0.000403       | down|
| MAPK signaling pathway      | FGF7     | hsa-miR-30e-5p(hsa-mir-30e)    | 0.012066       | up   |
|                             | FGF7     | hsa-miR-30c-5p(hsa-mir-30c-2)  | 0.026564       | up   |
|                             | FGF7     | hsa-miR-30c-5p(hsa-mir-30c-1)  | 0.025462       | up   |
| MAPK signaling pathway      | NGF      | hsa-let-7e-5p(hsa-let-7e)      | 4.10E-08       | up   |
| PI3K-Akt signaling pathway  | MAP3K13  | hsa-miR-206(hsa-mir-206)       | 0.022004       | down|
|                             | LPAR6    | hsa-miR-24-3p(hsa-mir-24-2)    | 8.77E-07       | up   |
|                             | LPAR6    | hsa-miR-24-3p(hsa-mir-24-1)    | 9.25E-07       | up   |
|                             | PDGFC    | hsa-let-7d-3p(hsa-let-7d)      | 0.020946       | up   |
|                             | CCND1    | hsa-miR-6087(hsa-mir-6087)     | 2.36E-19       | up   |
|                             | NGF      | hsa-let-7e-5p(hsa-let-7e)      | 4.10E-08       | up   |
|                             | PIK3CD   | hsa-miR-7704(hsa-mir-7704)     | 0.000227       | up   |
|                             | VTN      | hsa-miR-4508(hsa-mir-4508)     | 0.001787       | up   |
| Term                        | GeneName | miRNA                        | log2FoldChange | stat |
|-----------------------------|----------|------------------------------|----------------|------|
| FGF7                        | hsa-miR-30e-5p(hsa-mir-30e) | 0.012066        | up             |
| FGF7                        | hsa-miR-30c-5p(hsa-mir-30c-2) | 0.026564        | up             |
| FGF7                        | hsa-miR-30c-5p(hsa-mir-30c-1) | 0.025462        | up             |
| GNGT2                       | hsa-miR-1246(hsa-mir-1246) | 3.44E-06        | up             |
| PI3K-Akt signaling pathway  | PPP2R5C  | hsa-miR-221-5p(hsa-mir-221) | 0.007438       | down |
|                             | BAD      | hsa-miR-1292-5p(hsa-mir-1292) | 0.009353       | down |
| EGFR-TKI resistance         | PIK3CD   | hsa-miR-7704(hsa-mir-7704)  | 0.000227       | up   |
|                             | PDGFC    | hsa-let-7d-3p(hsa-let-7d)   | 0.020946       | up   |
| EGFR-TKI resistance         | BAD      | hsa-miR-1292-5p(hsa-mir-1292) | 0.009356       | down |
| Cell cycle                  | CCND1    | hsa-miR-6087(hsa-mir-6087)  | 2.36E-19       | up   |

As mentioned above, exosomes contain a large number of proteins, nucleic acids, and lipids, which transmit information between cells[27]. Tumor-derived exosomes can be detected in patients' blood or other body fluids. They carry a large number of tumor-derived molecules[10], which can change the molecular phenotype to promote tumor progression and affect tumor microenvironment reconstruction[28]. This also shows that tumor-derived exosomes can be objects of liquid biopsy, reflecting the tumor burden and drug resistance. It has been proved that exosomes can affect the therapeutic response and induce drug resistance of tumor cells[29]. Recent research have suggested that drug-resistant cells transfer drug resistance to drug-sensitive cells through miRNAs and drug efflux mercury[28].

MicroRNAs (miRNAs), as a short non-coding RNA, have been thoroughly studied in the field of tumors[30]. It can inhibit or relieve protein expression by binding with target mRNA, so as to regulate the occurrence, transformation and drug response of tumor. When miRNAs are loaded into exosomes, they can be protected from degradation by RNases[31]. An interesting surprise was the discovery that exosome miRNAs could assist in the diagnosis of non-small cell lung cancer. We compared the levels of exosomes miR-184, miR-3913-5p, miR-3614-5p and miR-4746-5p in the serum of NSCLC patients and healthy people, and found significant differences between the two. The AUC of miR-184 was 0.803 (95% confidence interval: 0.701–0.905), and the area under the curve (AUC) was greater than 0.75, indicating that miR-184 of serum exosomes of lung cancer patients could be used as a biomarker for the diagnosis of NSCLC (Supplementary Fig. 3).
In the study of exosomal resistance, Chen [32] et al. found that miR-222-3p in exosomes can be used as a biomarker for gemcitabine resistance. Qin [33] et al. found that exosomes control DDP resistance by transmitting miR-100-5p. Exosome miRNAs and EGFR-TKI resistance have recently been published. Yan Zhang [34] et al. confirmed that exosomes miR-214 from Gefitinib-resistant PC9-GR could inhibit apoptosis in vitro, in vivo inhibit tumor growth and consequently gain Gefitinib resistance in EGFR-mutant lung cancer. Liu [29] et al. found that miR-522-3p in exosomes from H1975 cells can increase the resistance of Gefitinib to PC9 cells.

However, no published literature has discussed on the relationship between three-generation EGFR-TKI resistance and exosomes. Our study used next-generation sequencing to compare exosomes miRNAs in H1975 and H1975-OR cells in vitro, combined with miRNAs from three pairs of serum exosome of patients before and after drug resistance, and verified in another 64 NSCLC patients’ serum samples. We found that miR-184 and miR-3913-5p were significantly elevated in exosomes after Osimertinib resistance. Previous studies have shown that EGFR-independent mechanisms of Osimertinib resistance include bypass activation of PI3K pathway, which can be achieved through PIK3CA mutation/amplification and PTEN deletion. The sequencing results in the exosomes of H1975-OR cell supernatants confirmed that exosome PI3K/Akt pathway activation was involved in Osimertinib resistance (Fig. 6).

In the clinicopathological features, LDH has been proved to be closely related to clinical prognosis in a variety of malignant tumors [35]. NSCLC patients with higher LDH levels have a worse prognosis and shorter survival [36]. Although exosome miR-184 found in our study was related to LDH level (p = 0.018), due to the time limit of this experiment, we could not continue to follow up to obtain survival data, whether exosome miR-184 could be a prognostic indicator is not known. CEA is the most common biomarker of lung adenocarcinoma [37]. It has been confirmed that the increase of CEA level during TKI treatment for EGFR mutation patients may be a more sensitive predictor of the explosive progression of lung adenocarcinoma [38]. Platelet count (PLT) is often associated with platelet-to-lymphocyte ratio (PLR) [39]. Studies have suggested that the preoperative PLT-PLR score could be of great significance in predicting the prognosis of patients with surgically resected NSCLC [40]. In this study, we found that Exo-miR-3913-5p was related to TNM stage (p = 0.045), PLT (p = 0.024), CEA (p = 0.045), distant metastasis (p = 0.049) and bone metastasis (P = 0.03). Moreover, AUC in the ROS curve was greater than 0.75, which further suggested that exosome miR-3913-5p level was associated with advanced progression of lung adenocarcinoma in patients with EGFR mutations during the use of TKI.

It could be seen from the Venn diagram analysis that miR-184 was found in the overlap of Treat1 (cell) and Treat4 (A patient with EGFR exon 19 del). MiR-3913-5p was found in the overlap of Treat3 and Treat4, and both patients were patients with EGFR exon 19 deletion. However, we found in these subgroup analyses that both miRNAs were more significant in patients with EGFR exon 21 L858R mutations. Previous studies have found that exon 19 deletion mutations (55.0%) have a higher rate of T790M resistance mutations than exon 21 L858R point mutations (37.3%) [41]. Recent studies have shown that the hazard ratio of survival benefit for Asian and L858R mutant populations is close to 1.00 in all people...
receiving Osimertinib treatment[6]. Our study found that these two exosomal miRNAs changed significantly in the L858R mutant population, which might indicate that exosomal miRNAs were mainly involved in Osimertinib resistance for patients with EGFR 21 exon mutation.

Previous studies have reported that nearly half of patients would lost T790M mutation at the time of progression on Osimertinib[42], and this loss may be related to the early resistance of Osimertinib. The loss of T790M mutation is not conducive to prognosis[43]. Plasma T790M levels may predict acquired resistance[44]. However, considering that Osimertinib is selective for EGFR sensitivity and T790M mutation, scholars believe that the emergence of T790M under Osimertinib treatment is not a drug resistance mechanism[16]. Our study found that exosomes miR-3913-5p changed significantly in T790M-positive patients, indicating that exosomes miR-3913-5p may be involved in the drug resistance mechanism of T790M-positive patients. Exosomes miR-184 and miR-3913-5p are likely to be important molecules for resistance transmission of Osimertinib.

There are also some limitations and deficiencies in our research. The number of patients validated in this study was 64, and the amount of collected specimens was insufficient. Only patient serum samples were used to extract exosomes, and no further humoral exosomes were used for verification. The clinicopathological characteristics of the patients were collected, but no survival analysis was performed due to the limitation of follow-up time. The predicted target genes and pathways will be verified in our subsequent experiments.

Our experiments further explain the mechanism by which exosomes are involved in Osimertinib resistance. This study helps to further expand the application of liquid biopsy in the field of clinical lung cancer drug resistance. It will provide research directions for the design of a new generation of targeted drugs and exosome drug-loaded therapy for advanced NSCLC patients with EGFR mutations in the future.

Conclusions

Exosomes miRNAs derived from Osimertinib resistant strains are significantly different from sensitive strains. The mechanism of exosomes miRNAs involved in Osimertinib resistance is due to the activation of the bypass pathways (RAS-MAPK pathway abnormality, PI3K pathway activation). After patients obtained Osimertinib resistance, exosomes miR-184 and miR-3913-5p increased significantly in serum. These two exosomes miRNAs mainly cause Osimertinib resistance in lung cancer patients containing EGFR exon 21 L858R + and T790M+. They are regarded as important biomarkers of third-generation EGFR-TKI resistance. This not only enriches the application of liquid biopsy in lung cancer drug resistance, but also provides a direction for future targeted drug research.

Abbreviations

NSCLC: Non-small cell lung cancer; TKI: Tyrosine kinase inhibitor; EGFR: Epidermal growth factor receptor; BRAF: serine/threonine protein kinase b-Raf; T790M: Thr790Met; DMSO: Dimethyl sulfoxide; FBS: Fetal
bovine serum; PLT: platelet count; LDH: lactate dehydrogenase; CEA: carcinoembryonic antigen.

Declarations

Ethics approval and consent to participate

The research protocol was reviewed and approved by the Ethical Committee and Institutional Review Board of the Jinling Hospital affiliated to Nanjing University School of Medicine, and written informed consent was obtained from each patient included in the study.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

All the authors do not have any possible conflicts of interest.

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Authors' contributions

X.Li and T.Lv put forward the experimental idea. C.Chen completed the design of the overall experimental scheme. X.Li wrote the first draft of the article. Z.Wang, J.Liu and W.Sun completed the drawing of pictures and tables. M.Chen participated in the extraction of exosomes. X.Li, C.Chen and S.Zhu completed exosome identification and extended sample validation experiments. K.Shen and Y.Lv conducted data analysis. P.Zhan, F.Zhang, H.Liu conceived the project. T.Lv and Y.Song supervised the research. All the authors edited the final manuscript together.

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Isolation and identification of exosomes from cell supernatant. (A) The drug-resistant strain H1975-OR was obtained by continuous exposure to H1975 for 6 months. After incubating the two kinds of cells with different concentrations of Osimertinib for 72 hours, the cell viability was measured using the MTT method. (B) The average IC50 values of H1975 and H1975-OR were 4636 nM and 12101 nM, respectively, with significant differences (p=0.0215). (C) Observation of extracellular vesicles extracted from the cell supernatant by the transmission electron microscope, with a size between 30-150 nm. Scale = 200nm.
(D) Particle size analysis shows exosomal size distribution. (E) The expression of TSG101 and CD63 in supernatant-derived exosomes of the two cells was detected by western blot, and 20 μg of protein was loaded each.

Figure 2

Cluster analysis of miRNAs and target genes screened for differences in the exosomes of H1975-OR and H1975 cells. (A) Volcano map of differentially expressed miRNAs. Each point in the volcano map
represents a gene, and the red dots are significantly different genes that satisfy both \([\log FC]> 1\) and FDR < 0.05 threshold. The blue dots are only genes that meet FDR < 0.05, and the green dots are only genes that meet \([\log FC]> 1\). (B) Hierarchical cluster analysis of differentially expressed miRNAs. The vertical column represents the sample, each row represents a miRNA, the right side is marked with the miRNA name, and the color represents the expression (normalized read count is taken as log10), and the red to blue represents the gradually decreasing expression. (C) H1975-OR has elevated Top10 miRNAs (red) and down-regulated Top10 miRNAs (green) compared with H1975. (D) GO enrichment analysis of target genes for differential miRNAs. These target genes are divided into three categories: biological process, molecular function, and cellular component. List the number of genes (right) and proportions (left) involved in each pathway. (E) KEGG Pathway enrichment analysis of target genes for differential miRNA (histogram). The ordinate represents the name of the pathway, and the abscissa is the number of genes involved in each pathway. (F) KEGG Pathway enrichment analysis of differential miRNA target genes (dot plot). The ordinate indicates the name of the pathway, and the abscissa indicates the number of genes enriched in this pathway. The dot size indicates the size of the enrichment factor. The larger the point, the larger the enrichment factor. The larger the enrichment factor, the more prominent the annotation. A dot represents a pathway, and different colors indicate the size of the P value. The smaller the P value, the more significant the enrichment.
Figure 3

Differential miRNAs in plasma exosomes of patients before and after Osimertinib resistance. (A) Electron microscopy identification of patients' exosomes. The scales are 500nm, 200nm, and 100nm. (B) Particle size analysis and (C) Western Blot identification of exosomes. (D) Correlation analysis of exosome miRNA in patient serum and the cell supernatant. The correlation coefficient uses Pearson correlation test (column, row), and the color of each square corresponds to the correlation coefficient value. (E) Hierarchical cluster analysis of differentially expressed miRNAs in serum exosomes of 3 patients. Patient 1: Female, 53 years old, left lung adenocarcinoma stage IVB, EGFR 21 L858R mutation (Treat2 & Control2); Patient 2: Male, 62 years old, left lung adenocarcinoma stage IVB, EGFR 19 deletion (Treat3 &
MiR-184 and miR-3913-5p are elevated in exosomes after Osimertinib resistance. (A) Overlaid analysis of Wayne maps of exosomal miRNAs up-regulated and (B) down-regulated exosome miRNAs in the drug-resistant group than in the sensitive group. (C) q-PCR showed exosome miR-184 levels in patients with Osimertinib-resistant NSCLC (OR, n = 27) and patients without resistance (GR, n = 37), * p < 0.05. (D) Serum exosomal miR-3913-5p levels in patients with Osimertinib-resistant NSCLC (OR, n = 27) and sensitive patients (GR, n = 36), * p < 0.05. (E) Synthesis of the changes in the expression levels of the two exosomes miRNAs before and after resistance to Osimertinib. **p < 0.01. (F) Based on the above, draw ROC curves for miR-184 (green), miR-3913-5p (blue), and two miRNAs (red), respectively.
**Figure 5**

Exosomal miRNAs induce Osimertinib resistance targeting EGFR 21 mutations and T790M + patients. (A) q-PCR analysis of serum exosomal miR-184 levels and (B) miR-3913-5p levels in patients with drug resistance and sensitivity, in all patients with EGFR 19 exon deletion. (C) q-PCR analysis of serum exosomal miR-184 and (D) miR-3913-5p levels in patients with resistance and sensitivity in all patients with EGFR 19 exon L858R mutation. (E) (F) ROC curve of two miRNAs of serum exosomes in patients with EGFR 21 exon L858R mutation. (G) (H) is the level change of two miRNAs in T790M + patients measured using q-PCR.
Figure 6

Exosomal miRNAs participate in Osimertinib resistance mainly through bypass activation mechanisms in NSCLC

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