Study on RNA Regulating Iron Transport in Outer Membrane Vesicles of Hypervirulent *Klebsiella Pneumoniae*

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Abstract

**Background:** The iron acquisition ability of hypervirulent *Klebsiella pneumoniae* (hvKP) is an important part of its super virulence mechanism, increasing studies have proved that outer membrane vesicles (OMVs) are involved in the iron acquisition process of bacteria. Thus, we compared the difference in RNA expression in OMVs of hvKP in iron-rich and iron-deficient medium, and explore the possible mechanism of RNA in OMVs involved in hvKP iron acquisition.

**Results:** The results of high-throughput sequencing showed that in iron-deficient medium, there were 239 up-regulated and 89 down-regulated mRNAs in OMVs of hvKP, of which 20 mRNAs related to iron transport was up-regulated, mainly including siderophore synthesis and receptor genes, ATP binding cassette transporter family and iron sulfur cluster. Only two of the differential ncRNAs that regulate these mRNAs are up-regulated, which are IncRNAs.

**Conclusion:** We demonstrated that mRNA and IncRNA in OMVs were directly or indirectly involved in the iron acquisition mechanism of hvKP under iron deficiency environment, which enhanced the adaptive survival ability of hvKP. It provided a basis for further exploring the iron acquisition mechanism of OMVs involved in hvKP.

Background

*Klebsiella pneumoniae* is one of the pathogens causing hospital-acquired infections, which can be divided into classic *K. pneumoniae* (cKP) and hypervirulent *K. pneumoniae* (hvKP). In 1986, hvKP had been reported for the first time by Taiwanese [1]. HvKP mainly causes community-acquired infections, with the ability to infect healthy individuals of any age, the tendency to have multiple infection sites and subsequent metastatic spread, which is uncommon among other Enterobacteriaceae [2, 3].

Iron is an essential element for the growth and reproduction of microorganisms, while iron limitation is a common condition faced by pathogens inside the host. So, microorganisms have evolved various methods of obtaining iron. One of them is to produce siderophore with high affinity for iron ($K_M$ between $10^{25}$-$10^{52}$). Related research showed that the hypervirulent of hvKP is related to the formation of siderophore [4]. Recently, there have also been reports that outer membrane vesicles (OMVs) are involved in the bacterial iron acquisition process. *Mycobacterium tuberculosis* increases OMVs production in response to iron restriction and these OMVs contain mycobactin, which can serve as an iron donor [5]. In *Pseudomonas aeruginosa*, T6SS substrate TseF that incorporates into OMVs whereby it interacts with the iron-binding pseudomonas quinolone signaling (PQS) molecule to participate in iron uptake [6].

OMVs are double-layered spherical vesicles with a size of 20–250 nm released by Gram-negative bacteria. In addition to its role in bacterial adhesion to hosts, OMVs are also involved in stress responses, biofilm formation, inter- and intra-species delivery of molecules, antibiotic resistance, and modulation of host immune responses. Related studies have shown that the RNA contained in OMVs plays a role in the interaction between host and pathogen [7]. The sRNA in OMVs secreted by *P. aeruginosa* can weaken the
innate immune response in human airway epithelial cells and mouse lungs [8]. The microRNA of OMVs in actinomycetes can promote the production of TNF-α in human macrophages and cause neuroinflammatory diseases [9].

At present, there are few researches on the function and mechanism of RNA in OMVs and the virulence mechanism of OMVs involved in hvKP iron acquisition. Also, there were no related studies on K. pneumoniae. In the previous research, we have found that 17 proteins related to iron acquisition system in OMVs secreted by hvKP. In order to further explore the relationship between OMVs and hvKP iron acquisition capacity, this study performed RNA sequencing analysis on OMVs produced by hvKP cultured under iron deficiency and iron-rich conditions, aim to screen out differential RNAs related to iron acquisition, and analyze the mechanism of RNAs in OMVs involved in iron acquisition.

**Results**

**Bacterial growth curve.**

HvKP grew faster in both iron-deficient and iron-rich medium, and it grew slightly faster in iron-rich environments than in iron-deficient (Fig. 1). The morphology and physiological activity of the bacteria in the logarithmic phase are more typical. In order to obtain larger number of OMVs, sufficient amount of RNA, and maintain parallel between the two groups, we take hvKP bacterial solution at OD600 = 0.30 for subsequent experiment.

**Identification of OMVs.**

The OMVs of hvKP obtained after ultracentrifugation were negatively stained and the spherical vesicle-like structure could be seen under the transmission electron microscope. The size is between 20–250 nm, which is consistent with the size and shape of OMVs secreted by Gram-negative bacteria reported in other literatures (Fig. 2). At the same time, it could also find that more OMVs under iron-deficient.

**Differentially expressed mRNA and non-coding RNA (ncRNA).**

Heatmap can more visually see the differential expression of mRNA between the iron-deficient group and the iron-rich group (Fig. 3). Compared with the iron-rich group, the overall expression pattern of genes in the iron-deficient group was significantly different. In the iron-deficient group, most genes showed up-regulated expression patterns (red bands), while the iron-rich group was the opposite. The gene expression patterns of the two samples belonging to the iron-deficient group (A1 and A2) were similar, and the gene expression patterns of the two samples belonging to the iron-rich group (B1 and B2) were similar. This also proved that both the iron-deficient group and the iron-rich group have good sample repeatability.

Compared with the iron-rich group, we detected a total of 239 up-regulated mRNAs and 89 down-regulated mRNAs under iron-deficient medium. In addition, there were 20 up-regulated mRNAs related to iron transport, mainly including the siderophore synthesis and receptor genes, ATP binding cassette
transporter (ABC transporter) and iron-sulfur clusters. The specific description was shown in Table 1. At the same time, we also obtained 42 differential ncRNAs. In the iron-deficient environment, ncRNAs in OMVs secreted by hvKP, 34 of them were up-regulated and remaining 8 were down-regulated. Among these 42 ncRNAs, the corresponding ncRNAs that regulate the mRNA related to iron acquisition were found, and a total of 2 target ncRNAs were found: NC_006625.1 and NC_012731.1. Both of them were long non-coding RNA (lncRNA). Their corresponding target mRNAs are iroN, iutA, KP1_RS10680, proV and ygiD, as shown in Table 2. And these two differentially expressed lncRNAs were up-regulated under iron deficiency.
### Table 1
Differential mRNA related to iron acquisition in OMVs

| mRNA_id    | mRNA_name | mRNA-description                                                                 | log2FC  | Expression |
|------------|-----------|----------------------------------------------------------------------------------|---------|------------|
| KP1_3609   | iroN      | siderophore salmochelin receptor IroN                                             | 1.86245 | UP         |
| pK2044_01320 | iutA    | ferric aerobactin receptor IutA                                                   | 1.12960 | UP         |
| KP1_2278   | KP1_RS10680 | TonB-dependent siderophore receptor                                              | 1.79038 | UP         |
| KP1_1004   | fhuD      | iron(III) hydroxamate ABC transporter periplasmic binding protein                 | 1.68918 | UP         |
| pK2044_00160 | KP1_RS25940 | hypothetical protein                                                               | 1.43320 | UP         |
| KP1_1560   | dbhA      | 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase                                  | 2.09335 | UP         |
| KP1_1558   | entE      | 2,3-dihydroxybenzoate-AMP ligase                                                 | 1.12658 | UP         |
| KP1_0894   | leuC      | 3-isopropylmalate dehydratase large subunit                                       | 2.58790 | UP         |
| KP1_4614   | ygfZ      | tRNA-modifying protein YgfZ                                                       | 1.97479 | UP         |
| KP1_4312   | norW      | NADH:flavodox reductase                                                           | 1.20039 | UP         |
| KP1_3273   | btuC      | vitamin B12 ABC transporter permease BtuC                                         | 1.56476 | UP         |
| KP1_2332   | sapD      | peptide ABC transporter ATP-binding protein SapD                                  | 1.09891 | UP         |
| KP1_3794   | KP1_RS17655 | ABC transporter substrate-binding protein                                          | 1.37506 | UP         |
| KP1_4296   | KP1_RS20065 | metal ABC transporter substrate-binding protein                                  | 1.21127 | UP         |
| KP1_4271   | proV      | glycine betaine/L-proline ABC transporter ATP-binding protein ProV                | 1.02067 | UP         |
| KP1_3843   | yejF      | microcin C ABC transporter ATP-binding protein YejF                               | 1.25516 | UP         |
| KP1_1300   | ugpC      | sn-glycerol-3-phosphate ABC transporter ATP-binding protein UgpC                  | 1.79780 | UP         |
| KP1_1590   | KP1_RS7445 | ABC transporter permease                                                          | 1.94375 | UP         |
| KP1_2244   | KP1_RS10525 | ABC transporter permease subunit                                                  | 1.47212 | UP         |
| KP1_4734   | ygiD      | 4,5-DOPA dioxygenase exhadiol                                                      | 1.49341 | UP         |
Table 2
Differential ncRNA that regulates mRNA

| ncRNA_id                  | length | type | Target gene name | log\(_2\)FC | Expression |
|---------------------------|--------|------|------------------|-------------|------------|
| NC_006625.1:4383–4490(+)  | 107    | cis  | iroN             | 1.30879     | UP         |
| NC_006625.1:4208–4352(+)  | 144    | cis  | iroN             | 1.08899     | UP         |
| NC_006625.1:4498–4614(+)  | 116    | cis  | iroN             | 1.12811     | UP         |
| NC_006625.1:220959–221107(-) | 148   | cis  | iutA             | 1.12926     | UP         |
| NC_012731.1:2198124–2198227(-) | 103  | cis  | KP1_RS10680      | 1.28481     | UP         |
| NC_006625.1:32603–32709(+) | 106    | cis  | KP1_RS25940      | 2.06224     | UP         |
| NC_006625.1:26356–26502(+) | 146    | cis  | KP1_RS25940      | 1.44535     | UP         |
| NC_006625.1:38208–38329(-) | 121    | cis  | KP1_RS25940      | 2.30553     | UP         |
| NC_006625.1:38585–38740(-) | 155    | cis  | KP1_RS25940      | 2.08566     | UP         |
| NC_012731.1:4109193-4109294(-) | 101  | cis  | proV             | 2.35783     | UP         |
| NC_012731.1:4527117–4527261(-) | 144  | cis  | ygiD             | 1.62790     | UP         |

GO enrichment analysis and KEGG analysis of differentially expressed mRNAs and ncRNAs.

All differentially expressed mRNAs and ncRNAs were analyzed by GO classification. In general, GO analysis mainly counts the number of corresponding differential genes at three levels biological process, cellular component, and molecular function. We screened GO entries that were significantly enriched in differentially expressed genes. The larger the Rich Factor, the higher the degree of enrichment. It can be seen that processes related to transport activity were significantly enriched by GO analysis of differentially expressed mRNAs (Fig. 4 and Fig. 5). Multiple functions such as siderophore transport activity, siderophore and iron ion transmembrane transporter activity, iron chelation transport, iron assimilation and iron transport are all related to differentially expressed ncRNA.

Similarly, we can categorize the number of differentially expressed genes on each pathway from four aspects cellular process, environmental information processing, genetic information processing and metabolism. The TOP 30 signaling pathways of mRNAs and ncRNAs found were shown in Fig. 4 and Fig. 5. KEGG pathway analysis found that the siderophore group biosynthesis signal pathway (KO01053) was closely related to differentially expressed genes. From the KEGG pathway database, we can get a network diagram of the signal pathway (Fig. 6), from which we can see that the signal pathway involves pyochelin, mycobactin, Yersiniabactin, vibriobactin, enterochelin, bacillibactin, myxochelin and petrobactin. It could be found that the genes that affect this signaling pathway in this study were entE and dhbA that synthesize enterochelin. KEGG Pathway analysis of differential ncRNAs found that the bacterial secretion system pathways were related to iron acquisition capacity.
Discussion

In this study, RNA-seq of OMVs released by hvKP under iron-deficient and iron-rich conditions was designed to explore how mRNAs and ncRNAs contained in OMVs participate in the iron uptake mechanism of hvKP. This is the first study on the mechanism of iron acquisition in the OMVs involved in K. pneumoniae.

OMVs are one of the research hotspots of microbial neighborhood in recent years, which are considered to be closely related to the virulence and pathogenicity of bacteria. In Acinetobacter baumannii, OMVs can provide more protection to the existing defense system under antibacterial pressure, and OMV-mediated cytotoxicity of host cells and production of OMVs can be regulated by the BfmRS two-component system [10, 11]. OMVs can also be produced by bacteria in internal and external environments, but the production of OMVs is a secretion process controlled by the environment [12], so different OMVs extraction methods have a certain impact on the number and content of OMVs. The method used to extract OMV in this study is the most commonly used ultracentrifugation method. Our previous study used Stewart phospholipid analysis to quantitatively determine OMVs found that hvKP released more OMVs when grown in iron-deficient medium than in iron-rich, and the difference was statistically significant. It can also be observed under transmission electron microscope that OMVs produced under iron-deficient conditions are more than those under iron-rich conditions. From this we can preliminarily speculate that iron deficiency can stimulate the secretion of OMVs, which is consistent with the results in M. tuberculosis [2].

OMVs secreted by bacteria contain abundant nucleic acid, and mRNA in them carries a lot of genetic information. Differential mRNAs screening was performed on OMVs secreted by hvKP in iron-deficient and iron-rich medium in this study, it was found that hvKP had up-regulated expression of 20 iron transport-related mRNAs in iron-deficient environments. dhbA and entE, two of the genes involved in iron carrier synthesis, are located in the signal pathway of enterobactin synthesis through KEGG pathway analysis. Enterobactin is a catechol-type siderophore, which is produced by a variety of Gram-negative bacteria, including E. coli and K. pneumoniae, and has the strongest iron-affinity siderophore. It has been proved to promote iron acquisition and transshipment in a variety of Enterobacteriaceae [13–15].

When siderophore binds to the corresponding receptor, then directly releases the carried Fe\(^{3+}\) to the bacterial cytoplasm for growth. The siderophore receptor-associated gene iroN, which was up-regulated in this study, is a receptor for the iron phosphate bisphenol complex. GO analysis showed that iroN participates in the iron acquisition and transport in a variety of ways, one is involved in the transport of enterobactin; the second is involved in the steady state process of iron ions in the cell, KP1_RS25940 also participates in this process [16]. And the third is directly involved in the transport of iron. Another up-regulated siderophore receptor gene, iutA, is a gene encoding a specific outer membrane protein receptor for aerobactin. Its expression depends on the iron content in the environment. It has been reported that the addition of iron chelating agents to the culture medium increased the synthesis of iutA, and the growth of bacteria was not affected. When the iron was sufficient, the synthesis of iutA could be reduced.
During the infection of enteropathogenic and avian pathogenic *E. coli*, the expression of *iutA* increased, which was considered to be highly closely related to the pathogenicity of bacteria [18, 19].

There are also ten mRNAs related to ATP-binding cassette transporters (ABC transporter) and two mRNAs related to iron-sulfur clusters were up-regulated in the iron-deficient environment. ABC transporter is a transmembrane protein. It participates in the transport of various ions in the form of active transport. Iron-sulfur clusters participate in various biological processes, including iron storage. When iron is lacking, it can be used as iron donor. According to related reports, the expression of iron-sulfur clusters under iron-deficient environment would increase [20, 21].

lncRNA is usually with a length of more than 200 nucleotides and can interact with the target mRNA, thereby affecting and regulating the expression of mRNA [22, 23]. From the current situation, there is no report on lncRNA in bacterial OMVs, and there are only few reports focus on the function of sRNA in bacterial OMVs [24]. Among the ncRNAs involved in the regulation of differential mRNA related to iron uptake described above, a total of two differential lncRNAs were screened NC_006625.1 and NC_012731.1. And they all act on the target genes in the manner of cis regulation, indicating that they exist in the adjacent flanking sequences of *iroN*, *iutA*, KP1_RS10680, *proV*, and *ygiD* to affect gene expression. From our experiments, we can prove that these two lncRNAs affect the expression of iron transport-related mRNA by up-regulating their own expression, thus hvKP can break the iron limit under iron-deficient environment to enhance its ability to adapt to the environment.

Through KEGG pathway analysis of ncRNA, we can find a signaling pathway of bacterial secretion system. It has been reported that Type III secretion system (T6SS) acts on OMVs and recruits iron through OMVs to enhance the virulence and infectivity of *P. aeruginosa* [6]. And in our previous research, we found that there were 6 kinds of T6SS-related proteins in hvKP OMVs. The results of this study further proved that the "T6SS-OMVs-Fe" network model, the three mechanisms work together to participate in the hvKP iron acquisition mechanism is possible.

**Conclusions**

In summary, this study successfully extracted OMVs of hvKP and found up-regulated mRNAs related to iron acquisition in the OMVs of hvKP in iron-deficient environments, including siderophore biosynthesis, siderophore receptor, ABC transporters, and iron-sulfur clusters. Also found that there are two lncRNAs that regulate these mRNAs as target genes. This study laid the foundation for further exploration of the iron regulation mechanism of OMVs involved in hvKP. At the same time, this research may provide ideas for targeting design of RNA antagonists or inhibiting the production of OMVs to reduce the virulence of hvKP, opening up new treatment methods and preventing infection for hvKP.

**Methods**

*Strain cultivation and growth curve.*
HvKP standard strain TW513 was donated by Taiwan University. After isolating and purifying the strain, it was added to physiological saline and ground into a bacterial suspension with a concentration of 0.5 McFarland Standard (McF). Iron deficiency medium was prepared using standard M9 basal medium powder. An iron-rich medium with a FeCl₃ concentration of 2.0 µg/ml was prepared by adding FeCl₃·6H₂O to the Iron deficiency medium. The bacterial suspension was added to the iron-deficient medium and the iron-rich medium at a ratio of 1:100. Cultures were grown at 37 °C with agitation (200 rpm), and growth was determined by spectrophotometry at 600 nm.

**Extraction, purification and identification of OMVs.**

After the bacterial suspension and medium were cultured to the logarithmic phase of growth (determined according to the results of the growth curve), centrifuged at 3800 g and 4 °C for 15 min, then filtered the supernatant with a 0.45 µm disposable sterile filter (Millipore, America). Then used an ultracentrifuge Optima XPN (BECKMAN COULTER, America) to centrifuge at 100,000 g and 4 °C for 3 h, and kept the pellet to resuspend in sterile PBS buffer. Centrifuged again at 100000 g at 4 °C for 2 h, added 500µL of sterile PBS buffer to resuspend the pellet, and placed it at -80 °C for use. Observed its morphology and size using a transmission electron microscope (Philips, Netherlands).

**RNA sequencing (RNA- seq) and results analysis.**

miRNeasy Mini Kit (QIAGEN, Germany) were used for RNA extraction and purification. The purified Total RNA was ligated with a 3’ linker and a 5’ linker, and then reverse transcription, amplification, cDNA library size selection, purification, and finally the construction of the sequencing sample library was completed. Used the Qubit® 2.0 Fluorometer to detect the concentration of the library, and the Agilent 2100 to detect the size. Single-end sequencing was performed using the Illumina HiSeq sequencer (Illumina, America). The data collection software provided by Illumina was used for real-time data analysis during the sequencing process.

**GO enrichment analysis and KEGG analysis of differential RNA.**

EdgeR was used to analyze the differentially expressed genes, in the iron-deficient group and the iron-rich group, and the p and q values were obtained. At the same time, we calculate the differential expression multiple based on the FPKM value, that is the FC value. With \(|\logFC|>1\) and \(q\) value < 0.05 as the rule, the differentially expressed genes between two groups were screened out. Differentially expressed genes used online analysis database (http://geneontology.org/) for GO enrichment analysis and online analysis software (www.kegg.jp) for KEGG enrichment analysis. The top 30 GO entries and KEGG pathways with the highest enrichment were selected.

**Abbreviations**

hvKP hypervirulent Klebsiella pneumoniae
OMVs outer membrane vesicles

$K_M$ Michaelis constant

RNA ribonucleic acid

mRNA messenger ribonucleic acid

ncRNA non-coding ribonucleic acid

IncRNA long non-coding RNA;

K. pneumoniae Klebsiella pneumoniae

cKP classic Klebsiella pneumoniae;

PQS Pseudomonas Quinolone Signaling

sRNA small ribonucleic acid

microRNA micro ribonucleic acid

ABC transporter ATP-binding cassette transporters

*M. tuberculosis* Mycobacteria tuberculosis

*E. coli* Escherichia coli

T6SS Type VI secretion system

*P. aeruginosa* Pseudomonas aeruginosa

McF McFarland Standard

RNA- seq RNA sequencing

cDNA complementary deoxyribonucleic acid

**Declarations**

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**Authors’ contributions**
Mao Zhou, Siyi Wang and You Lan designed the study. Mao Zhou and Siyi Wang performed experiments and wrote the main manuscript text. Xin Li, Xuan Liu and Jiahui Wang are responsible for acquisition, analysis of data Qun Yan and Jun Li approved the final version of this manuscript. Wenen Liu made overall planning and guidance. All authors read and approved the final manuscript.

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**Availability of data and materials**

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

**Ethical approval and consent to participate**

The study was approved by the Ethics Committee of Xiangya Hospital, Central South University. No informed consent was taken because this study was retrospective, and it did not cause additional medical procedure.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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Figures
**Figure 1**

Growth curve of HvKp strain under different iron content environment
Figure 2

OMVs of hvKp under transmission electron microscope. The picture on the left is under iron-deficient medium, and the right is under iron-rich medium.
Figure 3

Heat map of differential genes between iron-deficient and iron-rich group. A1, A2 represent RNA of OMVs in iron-deficient group; B1, B2 represent RNA of OMVs in iron-rich group. Red means up-regulated differential genes, and green means down-regulated differential genes. Each small square represents a gene, and its color indicates the expression level of the gene. Each row represents the expression level of each gene in different samples, and each column represents the expression level of all genes in each sample.
Figure 4

GO and PEGG analysis of differential mRNAs. a, statistical graph of GO function classification of differential mRNAs; b, scatter plot of GO enrichment of differential mRNAs; c, KEGG pathway classification statistics of differential mRNAs; d, scatter plot of KEGG enrichment of differential mRNAs.
Figure 5

GO and PEGG analysis of differential ncRNAs. a, statistical graph of GO function classification of differential ncRNAs; b, scatter plot of GO enrichment of differential ncRNAs; c, KEGG pathway classification statistics of differential ncRNAs; d, scatter plot of KEGG enrichment of differential ncRNAs.
Siderophore biosynthesis signal pathway. This signaling pathway involves Pyochelin, mycobactin, yersiniabactin, vibriobactin, enterochelin, and bacillibactin, myxochelin and special anthrax siderophore petrobactin. The orange boxes are the two differential genes that affect the signaling pathway in this study. This signaling pathway not only occurs in K. pneumoniae, but also plays a role in a variety of Gram-negative bacteria such as E. coli, Salmonella enterica, Enterobacter cloacae, and P. aeruginosa.