Integrated microRNA and mRNA Transcriptome Sequencing Reveals the Potential Roles of miRNAs in Stage I Endometrioid Endometrial Carcinoma

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Abstract

Endometrioid endometrial carcinoma (EEC) is the most dominant subtype of endometrial cancer. Aberrant transcriptional regulation has been implicated in EEC. Herein, we characterized mRNA and miRNA transcriptomes by RNA sequencing in EEC to investigate potential molecular mechanisms underlying the pathogenesis. Total mRNA and small RNA were simultaneously sequenced by next generation sequencing technology for 3 pairs of stage I EEC and adjacent non-tumorous tissues. On average, 52,716,765 pair-end 100 bp mRNA reads and 1,669,602 single-end 50 bp miRNA reads were generated. Further analysis indicated that 7 miRNAs and 320 corresponding target genes were differentially expressed in the three stage I EEC patients. Six of all the seven differentially expressed miRNAs were targeting on eleven differentially expressed genes in the cell cycle pathway. Real-time quantitative PCR in sequencing samples and other independent 21 pairs of samples validated the miRNA-mRNA differential co-expression, which were involved in cell cycle pathway, in the stage I EEC. Thus, we confirmed the involvement of hsa-let-7c-5p and hsa-miR-99a-3p in EEC and firstly found dysregulation of hsa-miR-196a-5p, hsa-miR-328-3p, hsa-miR-337-3p, and hsa-miR-181c-3p in EEC. Moreover, synergistic regulations among these miRNAs were detected. Transcript sequence variants such as single nucleotide variant (SNV) and short insertions and deletions (Indels) were also characterized. Our results provide insights on dysregulated miRNA-mRNA co-expression and valuable resources on transcript variation in stage I EEC, which implies the new molecular mechanisms that underlying pathogenesis of stage I EEC and supplies opportunity for further in depth investigations.

Introduction

Endometrial cancers are the most frequent cancer occurring in the female genital tract, and its incidence has increased in recent years [1]. Endometrioid endometrial carcinoma (EEC) is the most dominant subtype, accounting for ~80% of cases [2]. To date, most clinical trials of chemotherapeutics for advanced and recurrent EEC have shown limited benefits [3,4] and information regarding the molecular mechanisms of EEC etiology is still limited. Searching for innovative diagnosis markers and therapeutic targets for EEC is thus necessary.

Aberrant transcript expression levels are commonly observed in cancer and these aberrations could alter biological pathways and disease phenotypes. Next-generation sequencing (NGS) of RNA (RNA-seq) is a powerful tool to investigate the comprehensive transcriptome. RNA-seq has greatly enhanced our knowledge of the transcriptome in cancer [5] recently. MicroRNAs (miRNAs) are a class of small non-coding RNAs that can regulate mRNA expression and control various biological processes [6] through binding to the 3’ untranslated region of mRNAs. It is reported that about 30% of human genes [7] and virtually all cellular processes are regulated by miRNAs [8]. Studies on miRNA dysregulation in cancers have risen rapidly recently, including those in EEC. MI RNAs such as hsa-miR-503, hsa-miR-205, and hsa-miR-200b are dysregulated in EEC and they could regulate cell proliferation, differentiation, apoptosis, and carcinogenesis [9–11]. However, studies on concurrent transcriptome characterization of both mRNA and miRNA in EEC are still lacking.

In this study, we applied an integrative approach by simultaneously sequencing both miRNA and mRNA for International Federation of Gynecology and Obstetrics (FIGO) Stage I EEC and adjacent non-tumorous tissues to investigate the mechanisms responsible for the pathogenesis of EEC. Quantitative real-time reverse transcription PCR (qRT-PCR) in other independent
patients was performed on the selected miRNA and mRNAs. Transcript sequence variants such as single nucleotide variant (SNV) and short insertions and deletions (Indels) were also characterized. Our investigation may shed light on the molecular pathogenesis of EEC and offer new possibilities for early diagnosis and systemic treatment.

Materials and Methods

Ethics Statement

Our study design received approval from the institutional review board of the third affiliated hospital of Guangzhou medical college (Guangzhou, China). Written informed consent was obtained from all patients.

Sample collection and RNA extraction

Three female patients aged from 32 to 47 were diagnosed as FIGO stage I EEC (two stage IA patients and one stage IB patient) in the third affiliated hospital of Guangzhou medical college. All primary tumor and adjacent non-tumorous samples were obtained from these three patients who underwent surgical tumor resection. All samples were frozen at −80°C until RNA extraction. Total RNA was isolated by using RecoverAll Total Nucleic Acid Isolation Kit (Life Technologies, Carlsbad, CA, USA) for mRNA and miRNA sequencing, according to the manufacturer’s instructions. Integrity of RNA was checked by Agilent 2100 bioanalyzer.

Sequencing

The sequencing library was prepared according to the standard protocol. Briefly, for mRNA sequencing, total RNA was firstly poly-A-selected followed by fragmentation of RNA into small pieces. The cleaved RNA fragments were reverse transcribed to cDNA end-repaired and ligated with Illumina adapters using Quick ligation TM kit (NEB) and DNA ligase. The libraries were then fractionated on agarose gel; 200-bp fragments were excised and amplified by PCR. After purification, the quality of libraries was checked by using Bioanalyzer 2100 (Agilent). MRNA sequencing was then performed on an Illumina HiSeq 2000 sequencer with 100 bp pair-end reads. For small RNA sequencing, libraries were prepared by ligating different adaptors to the total RNA followed by reverse transcription and PCR amplification. Small RNA libraries were sequenced on the Illumina HiSeq 2000 sequencer with 50 bp single-end reads, according to the standard manufacturer’s protocol. All raw data have been deposited in the NIH Short Read Archive database (SRP045645).

Table 1. Summary of mRNA sequencing.

| Sample id | Clean reads | Mapped reads | Uniquely mapped reads | Paired mapped reads | Unmapped reads | Transcript coverage (%) |
|-----------|-------------|--------------|-----------------------|--------------------|---------------|-------------------------|
| S1_N      | 53,204,178  | 47,891,621   | 45,603,601            | 44,892,334         | 5,312,557     | 40.63                   |
| S1_T      | 54,527,132  | 49,915,956   | 47,689,962            | 47,068,014         | 4,611,176     | 42.46                   |
| S2_N      | 52,006,058  | 47,807,313   | 46,016,910            | 45,189,796         | 4,198,745     | 40.82                   |
| S2_T      | 52,254,225  | 48,152,928   | 45,232,887            | 45,521,896         | 4,101,297     | 40.51                   |
| S3_N      | 52,891,237  | 48,016,297   | 45,448,760            | 45,323,990         | 4,874,940     | 40.61                   |
| S3_T      | 51,417,759  | 46,261,459   | 43,257,947            | 43,832,254         | 5,156,300     | 38.94                   |
| Average   | 52,716,765  | 48,007,596   | 45,541,678            | 45,307,714         | 4,709,169     | 40.66                   |

QRT-PCR of miRNA and mRNA expression

Expression of selected miRNAs and mRNAs were validated by qRT-PCR. Another 21 pairs of samples from stage I EEC patients (12 stage IA and 9 stage IB patients) were collected in addition to the original three pairs of sequencing samples. For mRNA expression, 2 μg total RNA was reverse transcribed and QPCR was done in the real-time cyclers (Strata Gene MX3000P qPCR system). The relative fold changes were determined using the 2−ΔΔCt method with GAPDH as endogenous controls. For mRNA expression, 5 ng of total RNA per miRNA was reverse transcribed into cDNA with SYBR-Green PCR Master Mix (Takara). QPCR was done in real-time cyclers (Strata Gene MX3000P qPCR system). The relative expression levels were...
determined using the $2^{-\Delta\Delta C_t}$ method with U6 small nuclear RNA as endogenous controls.

Each reaction was done in triplicate. Pair wise t-test was used to determine the expression difference between the two groups.

**SNV/Indels detection**

SNV/Indels detection was carried out by using VarScan [19] based on the following filtering criteria: 1) depth of coverage over 5×; 2) variation frequency over 20%; 3) base quality over 20. Detected SNVs and Indels were then annotated by using ANNOVAR [20].

**Results**

**Overview of transcriptome sequencing results**

To reveal the transcriptome of EEC, we sequenced the mRNA and small RNA from the same total RNA samples of stage I EEC patients using Illumina HiSeq 2000 sequencer. From the six mRNA libraries, an average of 52,716,765 pair-end 100 bp clean reads was generated (Table 1). Six small RNA libraries were also sequenced and 11,669,602 single-end 50 bp clean reads were generated on average (Table 2).

A total of 1079 genes were differentially expressed, including 488 up regulated and 591 down regulated ones (Figure 1). Seven miRNAs were differentially expressed. Among them, hsa-miR-99a-3p, hsa-let-7c-5p, hsa-miR-196a-5p, hsa-miR-328-3p, hsa-miR-337-3p were down regulated while hsa-miR-181c-3p and hsa-miR-25-5p were up regulated (Figure 1).

**MiRNA target prediction and integration of mRNA and miRNA expression profiles**

We ran the three prediction algorithms: Targetscan [16], PITA [17], and miRanda [18] using the selected 7 miRNAs and 1079 genes as input. This resulted in 1098 miRNA-mRNA pairs, which were supported by at least two prediction algorithms. We further integrated the sequencing data into the predicted miRNA-mRNA pairs to validate the target pairs. A total of 438 target pairs were found inversely expressed, including 320 dysregulated genes.

**Functional enrichment**

Biological pathway and gene ontology (GO) enrichment were done for the 320 dysregulated genes. Pathway analysis identified 12 pathways overrepresented with dysregulated miRNA target genes. Overall, a genetic cluster summarizing the functions of Cell cycle (hsa04110) was found to have the highest relationship with EEC (Table 3). Considering the pathways involved in cancer development, we identified several significantly related pathways, including p53 signaling pathway, GnRH signaling pathway, Pathways in cancer and Gap junction (Table 3). In addition to these classical pathways, pancreatic cancer and thyroid cancer pathway were also overrepresented with dysregulated genes, implicating a common oncogenic basis. GO enrichment analyses indicated that 9 GO items were significantly enriched with the dysregulated genes (Table 4). Consistent with the pathway analysis, the most significant GO category is mitotic cell cycle (Table 4).

**QRT-PCR validation of miRNA and mRNA co-expression**

Our pathway analysis showed that the most significant pathway overrepresented with dysregulated miRNA target genes was the cell cycle pathway ($P = 1.21E-03$). Since human carcinogenesis is believed to result from uncontrolled cell proliferation and many molecules in the cell cycle pathway have been reported to be

| Table 2. Summary of miRNA sequencing. |
|--------------------------------------|
| ID | Clean reads | Unmapped reads | Mapped reads | RNA | snoRNA | rRNA | ID | Clean reads | Unmapped reads | Mapped reads | rRNA | snoRNA | rRNA |
|---|-------------|----------------|-------------|-----|--------|------|---|-------------|----------------|-------------|------|--------|------|
| S1 N | 12,426,115 | 12,385,299 | 1,533 | 36,994 | 1,594 | 1,553 | S1 T | 11,941,555 | 11,994,456 | 1,549 | 1,549 | 1,549 | 1,549 |
| S2 N | 10,957,967 | 10,971,133 | 11,726 | 1,146 | 312 | 312 | S2 T | 10,957,967 | 10,945,204 | 1,937 | 312 | 312 | 312 |
| S3 N | 14,584,595 | 14,522,078 | 60,098 | 635 | 1,784 | 1,784 | S3 T | 10,344,999 | 10,424,446 | 2,081 | 1,784 | 1,784 | 1,784 |
| S1 N | 11,616,994 | 11,616,994 | 1,147 | 49301 | 1,147 | 49301 | Average | 11,616,994 | 11,616,994 | 1,147 | 49301 | 1,147 | 49301 |
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associated with endometrial cancer [21,22], our findings prompted us to validate the expression of miRNAs and their potential target genes in the cell cycle pathway. As shown in Figure 2, among the significantly dysregulated miRNAs, 5 downregulated miRNAs (hsa-let-7c-5p, hsa-miR-196a-5p, hsa-miR-328-3p, hsa-miR-337-3p, and hsa-miR-99a-3p) were predicted to target genes encoding 10 upregulated cell cycle pathway-related mRNAs (E2F5, CDKN2A, CCNA2, TP53, BUB1B, CCNE1, CDK1, MCM4, SKP2, and CDC6). Additionally, one upregulated miRNA, hsa-miR-181c-3p, was predicted to target the downregulated TGFB3 gene in cell cycle pathway (Figure 2). All these 6 miRNAs and 11 genes were present in the three pairs of sequencing samples. Differential expression was confirmed for all the 6 miRNAs and 11 genes in sequencing samples and other independent 21 pairs of samples, as shown in Figure 3. These 6 miRNAs may therefore play a role in the pathogenesis of EEC.

In addition, for these 6 miRNAs and 11 mRNAs, synergistic regulations among the six miRNAs were detected (Figure 2).

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**Table 3.** Pathway analysis based on miRNA-targeted differentially expressed genes.

| KEGG id | KEGG description                  | KEGG subclass                      | p-value    |
|---------|-----------------------------------|------------------------------------|------------|
| hsa04110 | Cell cycle                        | Cell growth and death              | 1.21E-03   |
| hsa04115 | p53 signaling pathway             | Cell growth and death              | 1.44E-02   |
| hsa04912 | GnRH signaling pathway            | Endocrine system                   | 2.21E-02   |
| hsa04713 | Circadian entrainment             | Environmental adaptation           | 2.24E-02   |
| hsa05200 | Pathways in cancer                | Cancers                            | 3.41E-02   |
| hsa04973 | Carbohydrate digestion and absorption | Digestive system              | 3.94E-02   |
| hsa0480  | Glutathione metabolism            | Metabolism of other amino acids    | 4.03E-02   |
| hsa05212 | Pancreatic cancer                 | Cancers                            | 4.09E-02   |
| hsa04540 | Gap junction                      | Cell communication                 | 4.32E-02   |
| hsa04114 | Oocyte meiosis                    | Cell growth and death              | 4.65E-02   |
| hsa05216 | Thyroid cancer                    | Cancers                            | 4.72E-02   |
| hsa04918 | Thyroid hormone synthesis         | Endocrine system                   | 4.90E-02   |
example, E2F5 was predicted as a target of hsa-miR-196a-5p, hsa-miR-337-3p and hsa-let-7c-5p; TP53 was predicted as the target of both hsa-miR-328-3p and hsa-let-7c-5p; MCM4 was targeted by hsa-miR-196a-5p, hsa-miR-337-3p, and hsa-miR-99a-3p; SKP2 was targeted by hsa-miR-337-3p and hsa-let-7c-5p; CDK1 was targeted by hsa-miR-196a-5p and hsa-miR-337-3p.

Sequence variants in EEC transcriptome

Sequence variants analysis detecting SNVs and Indels was carried out using VarScan [19]. The results are summarized in Table 5. A total of 346 variants (17.06%) are from exonic regions of the genome while 1682 (82.93%) are from the non-protein coding regions. Around 75% (260/346) of the exonic variants, including 181 nonsynonymous SNVs, 14 stop gain variants and 65

| GO id         | GO description                  | GO class | p-value     |
|---------------|---------------------------------|----------|-------------|
| GO:0000278    | mitotic cell cycle              | Process  | 1.60E-03    |
| GO:0000777    | condensed chromosome kinetochore | Component| 1.17E-02    |
| GO:0007051    | spindle organization            | Component| 1.17E-02    |
| GO:0008156    | negative regulation of DNA replication | Process  | 1.17E-02    |
| GO:0032133    | chromosome passenger complex    | Component| 2.17E-02    |
| GO:0005876    | spindle microtubule             | Process  | 2.17E-02    |
| GO:0009636    | response to toxic substance     | Process  | 2.17E-02    |
| GO:0051301    | cell division                   | Component| 4.72E-02    |
| GO:0007067    | mitosis                         | Component| 4.72E-02    |

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Figure 2. MiRNA-gene network of the cell cycle pathway in endometrioid endometrial carcinoma. Protein symbols were marked according to gene expression pattern. Those with red frames are up regulated while those with blue frames are down regulated in endometrioid endometrial carcinoma tissues. Similarly, miRNAs with blue background were down regulated while the miRNA with red ground were up regulated in endometrioid endometrial carcinoma tissues.

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framshift Indels, would result in protein coding alterations. Moreover, 17 out of 260 protein-affected variants overlapped with the somatic mutations in the cancer (COSMIC) [23] (Table S1 and Table S2). Of the non-protein coding variants, around half (363/1682) are in the UTR region.

Discussion

Compared with traditional microarrays, NGS enables the identification of novel and more detailed studies of biological pathways in complex diseases. Here with mRNAs and small RNA sequencing for three pairs of stage I EEC and adjacent normal tissues, we provide a global overview of the stage I EEC transcriptome and reveal the miRNA regulation of transcript expression.

MiRNA data analyses identified 7 dysregulated miRNAs (Figure 1) and mRNA sequencing identified a total of 1079 differentially expressed genes (Figure 1). To gain insights into the targets of differentially expressed miRNAs, a stringent prediction of miRNA targets was made by using three different target prediction methods. We further integrated the sequencing data into the predicted miRNA-mRNA pairs to validate the target pairs. A total of 438 target pairs were found inversely expressed, which can serve as references for further studies on miRNA and its targets. Both GO and KEGG pathway enrichment analyses of the putative target genes revealed the dysregulation of the cell cycle process, suggesting that our results were in line with the basic tumor characteristics. We further validated the expression levels of miRNA-target pairs in the cell cycle pathway. As shown in Figure 2, 5 down regulated miRNAs (hsa-miR-196a-5p, hsa-miR-328-3p, hsa-miR-337-3p, and hsa-miR-99a-3p) were predicted to target genes encoding 10 upregulated cell cycle pathway-related mRNAs (E2F5, CDKN2A, CCNA2, TP53, BUB1B, CCNE1, CDK1, MCM4, SKP2, and CDC6). Additionally, one upregulated miRNA, hsa-miR-181c-3p, was predicted to target the downregulated TGFβ3 gene. All these 6 miRNAs have previously been suggested to be involved in human cancers. Downregulation of hsa-miR-196a has been identified in multiple cancer types, such as non-small cell lung cancer [24], pancreatic cancer [25], prostate cancer [26]. Moreover, let-7c was also found to be down-regulated in endometrial sarcomas when compared to the control samples [27]. Expression of miR-196a was also found to be significantly down-regulated in malignant melanoma cell lines and tissue samples [28]. Decreased expression of miR-328 was also found in multiple cancer types [29,30]. Loss of has-miR-337-3p expression has been associated with lymph node metastasis of human gastric cancer [31]. Downregulation of miR-99a was discovered in both plasma and tissues of endometrial cancer patients [32]. Overexpression of miR-181c has also been reported in other cancers, such as gastric cancer [33]. In the current study, we confirmed the involvement of hsa-let-7c-5p and hsa-miR-99a-3p in EEC and firstly found dysregulation of hsa-miR-196a-5p, hsa-miR-328-3p, hsa-miR-337-3p, and hsa-miR-181c-3p in EEC. Since all these miRNAs are dysregulated in stage I EEC, they may serve as potential diagnostic markers in clinical practice. In addition, considering the inhibition effects on cancer growth or metastasis of hsa-let-7c [24,26], hsa-miR-328 [29], hsa-miR-337 [31], these three miRNAs may provide new avenues for the prognosis and therapy for EEC. Among the target genes of these miRNAs in the cell cycle pathway, TP53 [34], CDK2A [34], SKP2 [35,36], CCNE1 [36], MCM4 [36], CDK1 [37] and TGFβ3 [38] have been extensively studied as anticancer targets in endometrial cancer. Overexpression of CCNA2 was reported to be an indicator of a poor prognosis endometrial carcinoma [39]. Currently, there is no report about the relationship between E2F5, BUB1B, or CDC6 and EEC. E2F5 is a transcription factor predicted as a target of hsa-miR-196a-5p, hsa-miR-337-3p and hsa-let-7c-5p. Human E2F5 gene is oncogenic [40] and overexpression of this gene was associated with worse clinical outcome in other cancers [41]. Both BUB1B and CDC6 are predicted as a target of hsa-let-7c-5p. BUB1B encodes a kinase involved in spindle checkpoint function and involvements of these two genes have been reported in other cancers [42,43]. Their biological functions in EEC need further study. Moreover, based on our validated co-expression relationships of these miRNA-mRNA pairs, further in vitro experiments may be carried out to further confirm that the differentially expressed miRNAs synergistically affect the expression of the predicted targets.

In addition, for the miRNAs and mRNAs we validated in the cell cycle pathway, synergistic regulations among the six miRNAs need further study. Moreover, based on our validated co-expression relationships of these miRNA-mRNA pairs, further in vitro experiments may be carried out to further confirm that the differentially expressed miRNAs synergistically affect the expression of the predicted targets.

In addition to expression analysis, we also analyzed the sequence variants. Besides proteins encoding SNV/Indels, a large number of variants are located in UTRs whose function still

Figure 3. QPCR expression on QPCR-validated genes and miRNAs in the cell cycle pathway. *, p<0.05; **, p<0.01.

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| Variants          | S1  | S2  | S3  | Total |
|-------------------|-----|-----|-----|-------|
| exonic            | 95  | 27  | 81  | 26    | 264  | 82  |
| synonymous SNV    | 26  | 0   | 26  | 0     | 74   | 0   |
| nonsynonymous SNV | 67  | 0   | 50  | 0     | 181  | 0   |
| stopgain          | 2   | 3   | 5   | 1     | 9    | 5   |
| frame shift insertion | 0  | 15  | 0   | 13    | 10   | 0   | 38  |
| frame shift deletion | 0  | 1   | 0   | 2     | 1    | 0   | 5   |
| non frameshift deletion | 0  | 8   | 0   | 7     | 12   | 0   | 27  |
| splicing          | 1   | 0   | 0   | 3     | 4    | 0   | 7   |
| UTR3              | 209 | 59  | 201 | 70    | 587  | 209 |
| UTR5              | 23  | 10  | 8   | 9     | 47   | 20  |
| downstream        | 28  | 4   | 30  | 3     | 74   | 9   |
| upstream          | 5   | 0   | 2   | 1     | 12   | 1   |
| intronic          | 185 | 19  | 189 | 27    | 404  | 50  |
| intergenic        | 114 | 11  | 92  | 8     | 244  | 25  |
| Total             | 659 | 130 | 603 | 145   | 1630 | 398 |

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remains unknown (Table 5). The analysis of SNV, Indels may provide possible targets for further mechanistic studies.

In summary, here we report a comprehensive characterization of miRNA and mRNA transcriptome in stage I EEC from expression level, miRNA/mRNA regulations and sequence variation. We integratively analyzed differentially expressed miRNAs and their target genes. Differential expression levels of target genes in the cell cycle pathway and their regulatory miRNAs were further validated in other independent samples. Our results will provide a valuable reference for future studies in transcript variation and regulation and will potentially be useful for mechanistic and preclinical studies.

Supporting Information

Table S1 Detail information of the identified single nucleotide variants. (XLSX)

Table S2 Detail information of the identified short insertions and deletions. (XLSX)

Author Contributions

Conceived and designed the experiments: QPJ GHX. Performed the experiments: HXZ QQL QLP JP. Analyzed the data: SYL FW ZTX JC HC. Contributed reagents/materials/analysis tools: XYX XXT. Contributed to the writing of the manuscript: HXZ QQL QPJ GHX.

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