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# Role of microRNA-16-5p, microRNA-194, IP-10 and APRIL in inducing inflammation in SARS-CoV-2 infected patients with severe symptoms

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The immune system induces pro-inflammatory conditions in the hospitalized SARS-CoV-2 infected patients. The roles played by humoral immunity-related factors in the pro-inflammatory conditions of the patients are yet to be clarified. It has been revealed that a proliferation-inducing ligand (APRIL), interferon (IFN)-γ-inducible protein 10 (IP-10) and microRNA-16-5p (miR-16-5p) play key roles in the induction of inflammation. Thus, in this study, we explored the expression levels of APRIL, IP-10, miR16-5p and miR-194 in the SARS-CoV-2 infected patients with severe symptoms. In addition, miR-194 can inhibit immune responses against viral infection. Further, we evaluated the expression of the molecule in the patients to explore the effect of the molecule during coronavirus disease 2019 (COVID-19). About 60 severe SARS-CoV-2 infected patients who were in the peak of the disease and 60 healthy controls were enrolled to evaluate APRIL, IP-10, miR16-5p and miR-194 expression levels. IP-10 expressions were evaluated using enzyme linked immunoassay (ELISA), while APRIL, miR-16-5p and miR-194 were evaluated using Real-Time PCR technique. The results showed that APRIL, miR-16-5p and miR-194 expression and serum levels of IP-10 significantly increased in the hospitalized SARS-CoV-2 infected patients compared to the healthy controls. There was a positive correlation between miR-16-5p and miR-194 expression levels in the patients. The significant participation of miR-16-5p in the induction of inflammation indicates its key role along with APRIL and IP-10 for excess inflammation in the hospitalized SARS-CoV-2 infected patients with severe symptoms. Upregulation of miR-194 may be natural negative feedback to the pro-inflammatory conditions and may be associated with establishment of SARS-CoV-2 infection.

**Keywords**: COVID-19, Humoral immunity

Humoral immunity plays key roles against viral infections, either in protection or eradication[1]. APRIL (a proliferation-inducing ligand) is an important molecule that participates in the maintenance and development of B lymphocytes, the main cells of the humoral immunity[2,3]. It is also a key agent for induction of self-reactive B cells, peripheral B-cell survival, and cluster of differentiation (CD) 40L-independent antibody isotype switching[3]. Further, APRIL significantly participates in the antibody glycosylation process[4], apart from the induction of inflammation during human pro-inflammatory disorders[5,6]. Therefore, APRIL may be considered as an important molecule in viral infections associated with the cytokine storm. In addition to APRIL, interferon (IFN)-γ-inducible protein 10 (IP-10), known as CXC ligand (CXCL)10, is an important chemokine that participates in several aspects of the immune responses, including humoral immunity[7,8].

Previous investigations revealed that several genetic and epigenetic factors regulate transcription and translation of the eukaryotic cells, such as immune cells[9]. The roles played by microRNAs (miRs), as the epigenetic factors, in the regulation of translation have also been documented by the investigators[10]. The miRs can increase or decrease stability of mRNAs and also translation from the molecules[11]. It has been reported that miR-16-5p is a key regulator of the humoral immunity[12,13]. Roles played by miR-16-5p in the regulation of expression of IP-10 and APRIL have been documented previously[14-16]. Accordingly, miR-16-5p by targeting the mRNA of the targeted molecules regulates the translation of them[14-16]. Therefore, the miRs can modulate humoral immunity via regulation of the expression of several molecules, including IP-10 and APRIL. In addition, it has been reported that some of

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the miRs play crucial roles in the inhibition of appropriate immune responses against viral infections. Wang *et al*.[17] reported significant inhibition of innate immunity against influenza H1N1 virus by miR-194. However, the potential targets for antiviral effects of the molecule are undiscovered. The coronavirus disease 2019 (COVID-19) patients with severe symptoms suffer from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, show upregulation of the above molecules.

COVID-19, which is induced by SARS-CoV-2, is associated with several complications, including pro-inflammatory related disorders[18]. The infection can affect many tissues, via over-activation of both humoral and cellular immunity[19]. Understanding of the main mechanisms behind severe symptoms in the SARS-CoV-2 infected patients, may help in finding appropriate therapeutic strategies. Therefore, in this study, we have made an attempt to evaluate expression levels of APRIL, IP-10 and miR-16-5p, as the main factors involved in the humoral immunity, and miR-194, as the inhibitor of immune responses against viral infections, in the hospitalized SARS-CoV-2 infected patients with severe symptoms and healthy controls.

## Material and Methods

### Subjects

In this project, 60 hospitalized SARS-CoV-2 infected patients who suffered from severe COVID-19 and were in the peak of the disease, and 60 healthy controls were registered to explore the expression of APRIL, IP-10, miR-16-5p and miR-194. The groups were not different regarding age and sex. Accordingly, the mean age of SARS-CoV-2 infected patients and healthy controls were 62.40 ± 3.733 and 57.50 ± 3.61 years old, respectively. The patients were hospitalized in Afzalipour Hospital, Kerman, Iran. The infections with COVID-19 were confirmed by the quantitative PCR test for COVID-19. The patients had respiratory distress, over 20 breaths per minute, low blood oxygen levels (<90%), more than 50% lung involvement, and need for intubation[20]. To overcome the interfered factors, the participants with infectivity with other viruses and bacteria, hypersensitivities, opium and immune suppressor drugs consuming, and smoking were excluded from the study. The blood samples were collected just before hospitalization and treatment. The informed consent form was completed by the all participants, and the project protocol was approved by the local ethical committee (IR.IAU.TMU.REC.1400.232).

### COVID-19 detection

For detection of COVID-19 infection, the viral RNA was purified using a commercial kit from Karmania Pars Gene Company, Kerman, Iran, according to the manufacturer's guidelines. The extracted viral-RNA was detected using a high-quality one-step COVID-19 Real-Time PCR kit (Karmania Pars Gene Company, Kerman, Iran) detected two genes viz. nucleocapsid (N) and RNA-dependent RNA polymerase (RdRp). The RNase P was detected simultaneously, as the internal control for Real-Time PCR.

### Evaluation of IP-10 serum levels

IP-10 serum levels were explored using commercial kits from Karmania Pars gene Company, Iran. Accordingly, 50 µL serum and standards were added to the pre-prepared ELISA plates and incubated for one hour. After washing the detection antibodies were added, and after 1 h of incubation and washing, HRP-avidin solution was added. Following incubation for 30 min and 5 times washing, the substrate was used, and the reactions were stopped after 15 min using the stopping solution for measuring the optical densities (ODs) at 450 nm in the BMP ELISA reader.

### RNA extraction and evaluation of APRIL, miR-194 and miR-16-5p

Total RNA and miRs were purified from blood samples using two distinct commercial kits from Karmania Pars Gene Company, Iran. Total RNA, miR-16-5p and miR-194 were converted to cDNA using cDNA synthesizes kit and specific cDNA synthesizes kit for miR-16-5p and miR-194, respectively (Karmania Pars Gene Company, Iran). To evaluate the levels of APRIL, miR-16-5p and miR-194, the commercial kits from Karmania Pars Gene, Iran was used in a Rotor-Gene Q thermal cycler (Qiagen, USA). Accordingly, the following program was used for APRIL: 3 min at 95ºC for 3 min for 1 cycle, 95ºC for 10 s, and 60ºC for 30 s for 45 cycles. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured as a housekeeping gene. The following program was used for measuring of the miR-16-5p and miR-194: 95ºC for 3 min for 1 cycle, 95ºC for 10 s, 58ºC for 30 s and 62ºC for 30 s for 45 cycles, and the U6 was measured as a housekeeping gene. The melt curve program was run for APRIL, miR16-P, and miR-194 Real-Time PCR, and the results were calculated using $2^{-\Delta\Delta Ct}$ formula[21].

**Statistical analysis**

Kolmogorov Smirnov test, under SPSS software version 16, revealed normal distribution of data. Accordingly, the differences between the hospitalized SARS-CoV-2 infected patients and healthy controls were evaluated using independent student t test. The Pearson correlation test was used to analyze the correlations among serum levels of IP-10 and relative expression of APRIL, miR16-P and miR-194 in the patients suffering from COVID-19.

## Results and Discussion

The results have shown significant increase in the mean relative expression of APRIL ($P$ =0.007), miR-16-5p ($P$ =0.007) and miR-194 ($P$ =0.017) of the SARS-CoV-2 infected patients compared to the healthy controls. As it is illustrated in Fig. 1, the mean relative expression of APRIL, miR-16-5p and miR-194 were 4.46 ± 1.07, 5.38 ± 1.50 and 11.23 ± 3.98, respectively in the SARS-CoV-2 infected patients and 1 ± 0.57, 1 ± 0.33 and 1 ± 0.68 in the healthy controls (Fig. 1).

The results also demonstrated that serum levels of IP-10 were significantly increased ($P$ <0.001) in the hospitalized SARS-CoV-2 infected patients (75.12 ± 9.08 pg/mL) when compared to healthy controls (11.31 ± 1.44 pg/mL). Figure 2 illustrates the serum levels of IP-10 in the patients and controls. Table 1 revealed that relative expression of APRIL, miR-16-5p and miR-194 had no significant correlations with serum levels of IP-10 in the severe COVID-19 patients. However, the levels of miR-16-5p had a positive correlation with miR-194 in the patients.

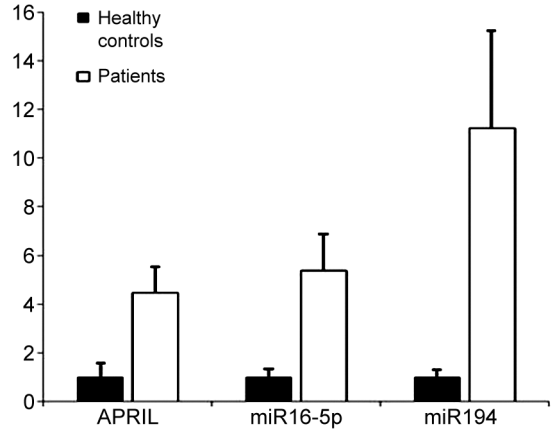


Fig. 1 — Relative expression of APRIL, miR-16-5p and miR-194 in the hospitalized severe COVID-19 patients and healthy controls. [The expression of APRIL ($P$ =0.007), miR-16-5p ($P$ =0.007) and ($P$ =0.017) had significantly increased in the hospitalized severe COVID-19 patients compared to healthy controls]

The results have demonstrated that the relative expression of miR-16-5p and APRIL and also serum levels of IP-10 were significantly increased in the hospitalized SARS-CoV-2 infected patients compared to the healthy controls. As mentioned previously, APRIL significantly participates in the development of humoral immunity[2,3]. As viral infections induce humoral immunity to produce antibodies against them, the coronavirus too induces humoral immunity via upregulation of APRIL. However, the relative expression of miR-16-5p was also increased, although it was not associated with the expression of APRIL. Due to the important roles played by miR-16-5p in the induction of inflammation[22,23], the increased expression of miR-16-5p could be a main mechanism for induction of the excessive inflammation in the SARS-CoV-2 infected patients with severe symptoms. Additionally, the severe COVID-19 patients suffer from excess inflammation[24,25]. The increased levels of miR-16-5p and IP-10 in the

Table 1 — Correlation among the levels of APRIL, miR-16-5p, miR-194, and IP-10 in the hospitalized COVID-19 severe patients

| | | APRIL | miR-16 | miR-194 | IP-10 |
|---|---|---|---|---|---|
| APRIL | Pearson correlation | 1 | –0.307 | –0.658 | 0.012 |
| | P value | - | 0.082 | 0.79 | 0.948 |
| miR-16-5p | Pearson correlation | –0.307 | 1 | 0.333 | –0.158 |
| | P value | 0.082 | - | 0.047 | 0.352 |
| miR-194 | Pearson correlation | –0.658 | 0.333 | 1 | –0.152 |
| | P value | 0.79 | 0.047 | - | 0.369 |
| IP-10 | Pearson correlation | 0.012 | –0.158 | –0.152 | 1 |
| | P value | 0.948 | 0.352 | 0.369 | - |

[Pearson correlation test revealed that the levels of miR-16-5p significantly had a positive correlation with expression of miR-194]

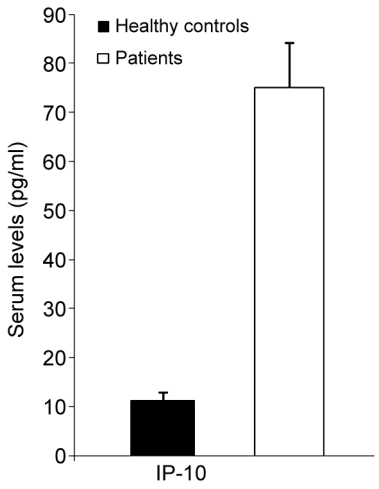


Fig. 2 — Serum levels of IP-10 in the hospitalized COVID-19 severe patients and healthy controls. [The serum levels of IP-10 ($P$ <0.001) had significantly increased in the hospitalized severe COVID-19 patients compared to healthy controls]

patients are possibly the main inducers of inflammation in the hospitalized SARS-CoV-2 infected patients with severe symptoms. As the increased miR-16-5p expression has no correlation with IP-10, the miR-16-5p-related inflammation is independent of IP-10. In aother words, miR-16-5p, by up- or down-regulation of pro- and anti-inflammatory molecules, respectively, may induce inflammation in the hospitalized severe COVID-19 patients.

Our above finding is in agreement with the earlier report that miR-16-5p can be considered as the main miR to target differentially expressed genes (DEGs) in response to SARS-CoV-2 infection[26]. The miR-16-5p can also induce inflammation via interactions with mRNA of immune system-related molecules, such as toll like receptors (TLRs)[22,23]. Additionally, human miRs can interact with viruses via direct binding to viral RNA. The mechanism is considered as important regulators of virus replication and pathogenesis[27]. Therefore, it may be hypothesized that miR-16-5p may directly bind to coronavirus and may modulate its replication and also pathogenesis, which needs to be further explored. A bioinformatic study predicted that miR-16-5p has a target on the SARS-CoV-2 genome[28]. The roles played by miR-16-5p on the replication and pathogenesis of SARS-CoV-2 are yet to be clarified and need more investigations, especially its direct effects on the immune system-related molecules in both *in vitro* and *in vivo* conditions.

The results of this study have also shown that miR-194 significantly increased in the patients. As mentioned previously, miR-194 plays inhibitory roles against antiviral functions of the immune system. Thus, upregulation of the molecule may be associated with the increased transfections of the viral infections to the non-infected cells and development of the disease. Therefore, it may be hypothesized that upregulation of miR-194 may be considered as an important mechanism for replication of SARS-CoV-2 in the patients with severe symptoms. However, expression of the molecule had no correlation with APRIL and IP-10. Accordingly, it appears that the inhibitory roles of miR-194 on the immune responses against SARS-CoV-2 are not dependent of APRIL and IP-10. Earlier studies have shown that miR-194 via downregulation of RORγt transcription factor, the main factor for development of Th17 lymphocytes, can regulate pro-inflammatory reactions in the SARS-CoV-2 infected patients[29,30]. The miRNA also can upregulate IL-10, the main anti-inflammatory cytokine, in the SARS-CoV-2 infected patients[31]. Thus, it suggests that miR-194 is an immune-regulator in the SARS-CoV-2 infected patients. Table 1 shows that the expression of miR-194 had a significant correlation with miR-16-5p. Due to the pro-inflammatory roles played by miR-16-5p, it appears that upregulation of miR-194 may be negative feedback to miR-16-5p to control immune responses and may be associated with non-appropriate immune responses against SARS-CoV-2. Therefore, it may be hypothesized that upregulation of miR-194 is a normal response to induce immune response homeostasis to control the severe inflammation and it may be the results of positive feedback of miR-16-5p on the half-life of miR-194. The hypothesis needs to be explored by further *in vitro* and *in vivo* investigations.

## Conclusion

The above results indicate that APRIL plays key role in SARS-CoV-2 infection and induces appropriate humoral immunity. The IP-10 as well as miR-16-5p may also significantly induce inflammation in the SARS-CoV-2 infected patients with severe symptoms. Upregulation of miR-194 may be a natural response to the pro-inflammatory conditions of the patients and also may be associated with non-appropriate immune responses against SARS-CoV-2.

## Conflict of interest

Authors declare no competing interests.

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