ABSTRACT: In order to study the effective mechanism of a traditional Chinese medicine (TCM), modified Jiu Wei Qiang Huo decoction (MJWQH), against H1N1-induced pneumonia in mice, we chose a holistic approach. A reverse-phase liquid chromatography with quadruple time-of-flight mass spectrometry (LC-Q-TOF-MS) was developed to determine metabolomic biomarkers in mouse serum for the MJWQH effects. Thirteen biomarkers of H1N1-induced pneumonia in mice serum were identified, which comprised L-valine, lauroylcarnitine, palmitoyl-L-carnitine, L-ornithine, uric acid, taurine, O-succinyl-L-homoserine, L-leucine, L-phenylalanine, PGF2α, 20-ethyl-PGE2, arachidonic acid, and glycerophospho-N-arachidonoyl ethanolamine. Among them, metabolites of amino acids, fatty acids and arachidonic acid had the most relevant changes in mice with H1N1-induced pneumonia. MJWQH effectively improved loss weight, lung index, biomarkers and inflammatory mediators such as prostaglandin E2 and phospholipase A2 in the infected mice. Importantly, MJWQH reversed the elevated biomarkers to the control levels from the infection, which provided a systematic view and a theoretical basis for its prevention or treatment. The results suggest that the protective effect of MJWQH against H1N1-induced pneumonia is possibly through regulation of pathways for amino acid, fatty acid and arachidonic acid metabolism. They also suggest that the LC-MS-based metabolomic strategy is a powerful tool for elucidation of the mechanisms of TCM. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: TCM; influenza virus; antiviral; metabolomics; biomarkers; pneumonia

Introduction

Infection caused by influenza viruses poses a considerable threat to human health around the world. It is necessary to develop drugs for the treatment of influenza infection. Traditional Chinese medicine (TCM) has played a very important role in the treatment of infectious diseases in China (Chang et al., 2011). Although in-depth studies on anti-viral, immunoregulation and cellular signaling pathways of TCM have been carried out, the precise mechanism of TCM is still unclear owing to the complexity of compounds generated through TCM. Therefore, understanding viral infection at a system level is of great interest to understand the protective mechanism afforded by TCM (Kitano, 2002).

The holistic approach is one of the most prominent characteristics of TCM for intervening and curing the dysfunction of living organisms (Wang et al., 2005). Logically, a holistic approach should be applied to explore the molecular mechanism of TCM. As a global analysis of nontargeted, low-molecular-weight metabolites from tissues or body fluids, metabolomics has been proven to be a valuable tool for evaluating the effect of TCM and elucidating its molecular mechanism (Dai et al., 2010; Xie et al., 2009; Zhao et al., 2012; Li et al., 2007).

Previous studies have identified important biomarkers such as arachidonic acid for the early diagnosis of H1N1 infection (Lu et al., 2012) and the tricarboxylic acid cycle, fatty acid biosynthesis and cholesterol metabolism during viral replication in the cell lines have also been examined (Janke et al., 2011; Lin et al., 2010). However, metabolomic profiles of H1N1-infected mice have not been studied and they might illuminate the disease process. Jiu Wei Qiang Huo (JWQH) decoction from the TCM monograph Cishi Nanzhiji of Jin Dynasty of China has been used for approximately 900 years for the treatment of influenza and viral pneumonia. A modified JWQH (MJWQH) was developed by Dr Yan Dexin, a ‘grandmaster of Chinese medicine’, granted by the Ministry of Health of the People’s Republic of China, which consists of six medicinal herbs including Notopterygium, Radix, Hebra Taraxaci, Radix Scutellariae, Radix Astragali, Rhizoma Atractylodis and Radix Saposhnikoviae. Radix Scutellariae is reported to exert inhibitory effects on influenza viruses, hepatitis B virus and HIV (Tseng et al., 2010; Dou et al., 2011; Zhang et al., 2012). Radix Notopterygii extract has anti-oxidant and anti-inflammatory activities against anti-influenza virus (Yang et al., 1991).

Abbreviations used: JWQH, Jiu Wei Qiang Huo; MJWQH, modified JWQH; PLS-DA, partial least squares-discriminate analysis; RT, retention time; TCM, traditional Chinese medicine.

Correspondence to: H. Zhu, Department of Biosynthesis, School of Pharmacy, Fudan University, Shanghai 201203, People's Republic of China. E-mail: hyzhu@mail.shcnca.cn

Department of Chinese Medicine, Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai 200092, People's Republic of China

Department of Biosynthesis, School of Pharmacy, Fudan University, Shanghai 201203, People's Republic of China

Institute of Chinese Medicine, Tongji University School of Medicine, Shanghai 200092, People's Republic of China

Research article

Hbea Taraxaci has anti-viral, antiseptic and anti-inflammatory activities (Zheng, 1990). Radix Astragali has anti-oxidant and immunopotentiating functions and anti-stress effects (Wang et al., 2009; Ye et al., 2010). Radix Saposhnikoviae has immunoregulatory and anti-oxidant activities (Zhang et al., 2008). Finally, Rhizoma Atractyloides has potent viral inhibitory and anti-oxidant effects (Inagaki et al., 2001).

MJWQH is widely used in the clinical practice of TCM for the treatment of influenza according to syndrome differentiation. Although individual components of MJWQH have shown anti-influenza virus activity, the effect of a combination of these six herbs has not been studied in vivo for its activity on the H1N1 virus. In the present study, a metabolomic method was developed in order to elucidate MJWQH effects in protecting against H1N1-induced pneumonia.

Experimental

Materials and reagents

The MJWQH was composed of the following six dried raw materials: 150 g of Radix Notopterygii (Notopterygium incisum Ting ex H.T. Chang), 600 g of Hbea Taraxaci (Taraxacum mongolicum Hand.-Mazz.), 300 g of Radix Scutellariae (Scutellaria baicalensis Georgi), 300 g of Radix Astragali (Astragalus membranaceus (Fisch.) Bge., Var. mongholicus (Bge.) Hsiao), 300 g of Rhizoma Atractyloides (Atractylodes lancea (Thunb.) DC) and 150 g of Radix Saposhnikoviae radix (Saposhnikovia divaricata (Turcz.) schischk.) These six herbs were purchased from Yanghetang Medicinal Materials Company (Shanghai, China) and authenticated before preparation by Professor. Zhihong Cheng, Department of Pharmacognosy, School of Pharmacy, Fudan University (Shanghai, China). Voucher specimens were deposited in the Herbarium Center Department of Pharmacognosy, School of Pharmacy, Fudan University. All of the raw materials were extracted by boiling water three times and then the decoction was dried in vacuo (70 °C) and ground into powder for use. The yield of the extraction was 24% (w/w).

High-performance liquid chromatography (HPLC) grade acetonitrile and formic acid were purchased from Merck (Dannstadt, Germany). Ultrapure water from a Milli-Q50 SP reagent water system (Millipore Corporation, MA, USA) was used for the preparation of samples and mobile phase.

Animal and virus strains

A murine-adapted strain of influenza virus (A/FM/1/47; H1N1) was obtained from the Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences (Beijing, China). Inbred male and female ICR mice, weighing 16–18 g were purchased from Shanghai Experimental Animal Center, Chinese Academy of Sciences, Shanghai, China [certificate no. SCXK (SH) 2012–0002] and maintained at the Animal Center of Fudan University. They had free access to food pellets and tap water under standard conditions of humidity (50 ± 10%), temperature (25 ± 2 °C) and a 12 h light–dark cycle. All animals were used in accordance with the policies and guidelines of the Ethics Committee for Animal Use in Fudan University.

Protective effect of MJWQH in mice afflicted by H1N1 pneumonia

Mice were randomly divided into the control and MJWQH treatment groups (16 mice per group) and intranasally infected with H1N1 virus. Infection dosage was given at 10 × LD50 (lethal dose) in a volume of 30 μL per mouse after anesthesia with isoflurane. The initial MJWQH treatment was given at a dosage of 5.6 g/kg intragastrically at 2 h post virus challenge then continued daily for 7 days. Mice in the normal control group were given saline alone under the same conditions. The experimental mice were observed for 7 days. Eight mice per group had body weight recorded and the survival rate was monitored daily; the other eight mice in the group were sacrificed to measure the lung index on day 4 post-infection, and serum were stored at –20 °C for analyses of inflammatory and metabolic mediators.

Measurement of inflammatory media in serum post-infection

Serum were collected by centrifugation and stored at –20 °C until assay. Concentrations of PGE2 and PL-A2 in serum were determined using specific ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions.
Sample preparation

For LC-MS analysis, 100 μL of mouse serum was extracted with 300 μL methanol. After vortex-mixing for 30 s, these samples were centrifuged at 12,000 rpm for 10 min to remove proteins. The supernatant was then transferred to autosampler vials. To ensure reproducibility throughout the analysis sequence, a quality control (QC) sample was prepared by pooling equal volumes (10 μL) of each serum sample and then preparing the pooled QC sample in the same way as the samples. The pooled QC sample was analyzed randomly through the analytical batch as the QC control. In addition, six aliquots of serum samples from one mouse were treated in the same process to assess the reproducibility of the sample preparation.

Conditions of liquid chromatography with quadruple time-of-flight mass spectrometry (LC-Q-TOF-MS)

Metabolomic analysis was performed using a Waters Acquity system (Milford, MA, USA) which was coupled to a Waters Micromass Q-TOF Premier mass spectrometer equipped with an electrospray ionization (ESI) source (Waters MS Technologies, Manchester, UK). The separation of all samples was performed on a Waters Acquity UPLC C18 column (1.7 μm, 2.1 × 100 mm) with the column temperature maintained at 40 °C. The flow rate was 0.4 mL/min and the mobile phase consisted of ultrapure water with 0.1% (v/v) formic acid (A) and acetonitrile with 0.1% (v/v) formic acid (B). The gradient program is shown in Table 1. The sample injection volume was 5 μL.

The mass spectrometer was operated in both positive and negative ion modes with parameters set as follows: drying gas (N2) flow rate, 8 L/min; gas temperature, 330 °C; source temperature, 100 °C; pressure of nebulizer gas, 35 psi; Vcap, 2.5 kV; skimmer, 65 V; cone gas flow, 50 L/h; desolvation gas flow, 700 L/h; scan range, m/z 50–1000. MS/MS data were acquired in the targeted MS/MS mode with a collision energy ranging from 5 to 20 V.

Method reproducibility

According to the different chemical polarities and mass values, the six extracted ions of mass 117.0784 (L-valine, positive mode), 343.2711 (lauroyl carnitine, positive mode), 399.3349 (palmitoyl-L-carnitine, positive mode), 125.01503 (taurine, negative mode), 131.09343 (L-leucine, negative mode) and 304.24009 (arachidonic acid, negative mode) were selected for assessment of its reproducibility. Extracts from six aliquots of a random serum sample were continuously injected to evaluate the reproducibility of samples extraction. The data acquired from six QC samples (three unknown samples injected per one QC sample) were used to assess LC-MS system reproducibility for the large-scale sample analysis. The peak areas and retention times (RT) of the six extracted ions were then used to determine the variation.

Data processing

The raw LC-MS data for all samples (excluding the data from blank samples) were initially processed by the Waters Masslynx Qualitative Analysis Software (Waters MS Technologies). The filter parameters were set as follows: restrict RT, 0.2–14 min; restrict mass, 80–1000 amu; peak relative height, ≥1.5%; mass tolerance, 0.05 Da; RT windows, 0.1 min. After Figure 2. Effect of MJWQH on inflammatory mediators. Mice were intranasally infected with 10 × 10^5 H1N1 influenza virus and sacrificed to collect blood and tissue on day 4 post-infection. (A) Changes in prostaglandin E2 (PGE2) and (B) phospholipase A2 (PL-A2) after infection. Each point or bar represents mean ± SD of 10 animals. * p < 0.05, **p < 0.01 compared with the VC group.

Table 2. The reproducibility and stability data for the proposed method

<table>
<thead>
<tr>
<th>Mode</th>
<th>Selected ions</th>
<th>Reproducibility (n = 6)</th>
<th>Peak area</th>
<th>Stability (n = 6)</th>
<th>Peak area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RT (min)</td>
<td>RSD (%)</td>
<td>Mean</td>
<td>RSD (%)</td>
</tr>
<tr>
<td>ESI+</td>
<td>117.0784</td>
<td>0.70</td>
<td>0.74</td>
<td>2690</td>
<td>8.56</td>
</tr>
<tr>
<td></td>
<td>343.2711</td>
<td>4.96</td>
<td>0.88</td>
<td>240</td>
<td>9.47</td>
</tr>
<tr>
<td></td>
<td>399.3349</td>
<td>6.63</td>
<td>0.96</td>
<td>1911</td>
<td>10.78</td>
</tr>
<tr>
<td>ESI−</td>
<td>125.01503</td>
<td>0.71</td>
<td>0.69</td>
<td>1703</td>
<td>9.65</td>
</tr>
<tr>
<td></td>
<td>131.09343</td>
<td>0.98</td>
<td>0.87</td>
<td>278</td>
<td>7.18</td>
</tr>
<tr>
<td></td>
<td>304.24009</td>
<td>5.92</td>
<td>0.93</td>
<td>2444</td>
<td>9.40</td>
</tr>
</tbody>
</table>

RT, Retention time; ESI, electrospray ionization.
filtration, a peak table was created which included information on the RT, and mass and ion intensity for all identified components. The data from each sample were then normalized to total area and all data were imported into the software SIMCA-P (version 11, Umetrics, Umea, Sweden) where multivariate analyses such as partial least squares-discriminate analysis (PLS-DA) were used for calculation.

Data analysis
All experimental data were expressed as means ± SD, and examined by one-way analyses of variance (ANOVA) with a Bonferroni correction and Dunnett’s t-test in SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA). p-Values less than 0.05 were considered significant.

Figure 3. Typical total ion chromatograms obtained from mice serum in the positive mode (A–C) and negative mode (D–F). Symbol: the control group (A and D), virus group (B and E) and MJWQH-treated group (C and F).

Table 3. Thirteen identification biomarkers detected by LC-Q-TOF-MS in both positive and negative ionization modes

<table>
<thead>
<tr>
<th>Mode</th>
<th>Number</th>
<th>RT (min)</th>
<th>Extract mass</th>
<th>Compound</th>
<th>Formula</th>
<th>Trenda</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESI (+)</td>
<td>1</td>
<td>0.70</td>
<td>117.0784</td>
<td>L-Valine</td>
<td>C_5H_11NO_2</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4.96</td>
<td>343.2711</td>
<td>Lauroylcarnitine</td>
<td>C_{19}H_{37}NO_4</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6.63</td>
<td>399.3349</td>
<td>Palmitoyl-L-carnitine</td>
<td>C_{23}H_{45}NO_4</td>
<td>↑</td>
</tr>
<tr>
<td>ESI (-)</td>
<td>4</td>
<td>0.66</td>
<td>132.0885</td>
<td>L-Ornithine</td>
<td>C_{5}H_{12}N_2O_2</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.71</td>
<td>168.0268</td>
<td>Uric acid</td>
<td>C_{5}H_{4}N_4O_3</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.71</td>
<td>125.0150</td>
<td>Taurine</td>
<td>C_{5}H_{11}NO_2</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.73</td>
<td>219.0763</td>
<td>O-Succinyl-L-homoserine</td>
<td>C_{8}H_{13}NO_6</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.98</td>
<td>131.0934</td>
<td>L-Leucine</td>
<td>C_{9}H_{11}NO_2</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>1.36</td>
<td>165.0775</td>
<td>L-Phenylalanine</td>
<td>C_{9}H_{11}NO_2</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4.57</td>
<td>354.2326</td>
<td>PGF2α</td>
<td>C_{20}H_{34}O_5</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>5.39</td>
<td>380.2515</td>
<td>20-Ethyl-PGE2</td>
<td>C_{22}H_{36}O_5</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>5.92</td>
<td>304.2401</td>
<td>Arachidonic Acid</td>
<td>C_{20}H_{32}O_2</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>5.94</td>
<td>501.2854</td>
<td>Glycerophospho-N-arachidonoyl Ethanolamine</td>
<td>C_{25}H_{48}NO_7P</td>
<td>↑</td>
</tr>
</tbody>
</table>

‘↑’ and ‘↓’ represent the compounds which were up- and down-regulated in the model group compared with the control group.
Results and discussion

Therapeutic efficacy in mice

The experimental mice were observed for 7 days. The infected mice began to lose weight from day 4, and all mice in the virus control groups died within 6 days (Fig. 1A). On the other hand, mice treated with MJWQH were well protected against the viral challenge and did not lose much weight compared with control mice. They all survived to day 7. Eight mice per group were sacrificed to measure lung index on day 4 post-infection, and serum were collected for the determination of PGE2 and PL-A2. In the virus group, the lung with severe congestion and edema led to the increased lung weight, which contributed to an increase in the lung index compared with the normal control (Fig. 1B). Serum concentrations of PGE2 and PL-A2 were significantly increased in the virus control group and inhibited by MJWQH treatment (Fig. 2A). MJWQH also alleviated the pulmonary damage induced by influenza virus (Fig. 2B).

LC-Q-TOF-MS method reproducibility

Calculated peak area and RT reproducibilities of the method are listed in Table 2. The RT precision for six selected ions in the extracts from six aliquots of the same serum sample was determined to be <0.96% relative standard deviation (RSD) in positive mode and 0.93% RSD in negative mode. In addition, the RSD values for peak areas of the extracted ions varied from 8.56 to 10.78% in the positive mode and 7.18 to 9.65% in the negative mode.

The data acquired from the QC sample also showed good system reproducibility. The RSD (%) of peak areas and RT of the extracted ions were 0.67–0.77% for RT and 6.47–10.15% for peak areas in the positive mode and 0.74–0.97 and 8.44–10.64%, in the negative mode, respectively. All results indicated that the established LC-MS method could be used for analysis of large-scale samples in the metabolomic studies.

Identification of biomarkers in H1N1-induced pneumonia in mice

Typical UPLC/MS total ion chromatograms of mice serum from the control group, virus group and MJWQH-treated group are shown in Fig. 3. Owing to a large number of signals obtained from the serum samples, a multivariate analysis method was needed to discriminate the ions which contribute to the classification of the control and model groups. Therefore, PLS-DA, a supervised projection method, was applied to the LC-MS data in this study. The established PLS-DA model could describe 99.4% of the variations in Y ($R^2_Y = 0.994$) with a predictive ability of 94.1% ($Q^2 = 0.941$) in the positive mode, and 99.9% of the variation in Y ($R^2_Y = 0.999$) with a predictive ability of 96.6% ($Q^2 = 0.966$) in the negative mode. In this model, a well-fitting PLS-DA model was established. As shown in Fig. 5, the score plot showed that the model group and the control groups could be separated clearly. Obvious separation between the control and model groups suggests that significant biochemical perturbation occurred in the model group.

To identify biomarkers in the model and control groups, the value of the variable importance in the projection (VIP) of the PLS-DA model was obtained. The t-test was used to reveal the significant differences of identified metabolites between the model and control groups. Therefore, potential biomarkers in H1N1-induced pneumonia in mice were studied. In this study, a total of 43 variables (the value of VIP > 1.0, $p < 0.05$) contributed to the metabolic variation. Among these variables, 13 (three in positive mode, 10 in negative mode) were identified by comparing the MS and MS/MS fragments with the metabolites found by searching in databases (http://metlin.scripps.edu, Figure 4). Intensity values for potential biomarkers in the control group, virus group and MJWQH-treated group. * $p < 0.05$ and ** $p < 0.01$ compared with the control group; # $p < 0.05$, ## $p < 0.01$ compared with the model group.
Biomarkers and their pathways

Among those 13 identified biomarkers in the mouse serum, two were depressed and 11 were elevated. The MJWQH treatment could significantly reverse of H1N1-altered biomarkers to the normal control levels (Fig. 4). The related pathway of every biomarker was identified by searching KEGG PATHWAY Database (http://www.genome.jp/kegg/). In these biomarkers, uric acid is related to purine metabolism; L-valine, L-ornithine, taurine, O-succinyl-L-homoserine, L-leucine and L-phenylalanine are related to amino acid metabolism; PGF2α, 20-ethyl-PGE2 and arachidonic acid are related to arachidonic acid metabolism; and lauroylcarnitine, palmitoyl-L-carnitine and glycerophospho-N-arachidonoyl ethanolamine are related to fatty acid metabolism.

When an animal is infected with a virus, body weight, body temperature and feeding are reduced, which is closely related to energy metabolism. Energy source is mainly generated by ATP substrate through the tricarboxylic acid cycle. Alternatively, amino acid, fatty acid and cholesterol catabolism also generate substrates for processing via the tricarboxylic acid cycle to produce energy. Therefore, based on our experimental results, disorder in both the amino acid metabolism and fatty acid metabolism might be associated with pathology of the infected mice. Among the biomarkers, lauroylcarnitine and palmitoyl-L-carnitine were abnormal forms of esterated carnitine, which indicated an energy disorder (Fabiak et al., 2011; Lehotay and Clarke, 1995; Sewell and Bohles, 1995).

Phospholipases are a widely distributed group of enzymes primarily implicated in the turnover of membrane phospholipids and lipid digestion. The PL-A2 family is known to be involved in lung inflammation and is also crucial for the inflammation pathways, as they are the first step for the production of eicosanoids and other inflammatory mediators (Melton et al., 2013). Therefore, it is conceivable that PL-A2 through either its pro-inflammatory role or the surfactant catabolism, might be involved in the pathogenesis of viral pneumonia (De Luca et al., 2011). When the host cells of mice are infected by the virus, it activates phospholipase A2 on the cell membrane and releases arachidonic acid from membrane phospholipids. Prostaglandin, thromboxanes and leukotrienes produced by arachidonic acid enzymes (lipooxygenase and cyclooxygenase) (Mizumura et al., 2003) lead to subsequent lung injury. Therefore, intervention of arachidonic acid metabolism could reduce the immunological injury and consequent host cell membrane damage during influenza virus infection.

In summary, the 13 identified biomarkers in H1N1-induced pneumonia not only revealed a new insight into the disease process of H1N1-induced pneumonia in vivo but also provided a theoretical basis for the prevention or treatment of pneumonia. The multiple pathways included in this study also demonstrated the complicated mechanisms of pneumonia.

Metabolomic study of MJWQH pre-treatment

The protective effects of MJWQH for the treatment of pneumonia could be obtained not only from the reducing pathology and inflammation, but also from metabolomic methods. The metabolite profiling using UPLC-TOF MS was applied to simultaneously monitor multi-metabolic pathways affected by H1N1 virus. The score plot of the PLS-DA model (Fig. 5) showed that the control, model and MJWQH pre-treatment groups were separated clearly, and the MJWQH pre-treatment group was closer to the control group than the model group, which suggested that MJWQH could inhibit the pathological process of pneumonia.

Conclusion

In this study, 13 metabolites were identified as potential biomarkers in H1N1-induced pneumonia in mouse serum. Arachidonic acid, fatty acid and amino acid metabolism were the main perturbed pathways in this pathological process. These biomarkers also provided a theoretical basis for the prevention or treatment of H1N1-induced pneumonia. Since MJWQH could reverse the pathological changes through regulation of these metabolites, this suggests that the LC-MS-based metabolomic strategy is a powerful approach for elucidation of the mechanisms of TCM.
Acknowledgments

This work was supported by the National Natural Science Foundation of China (grant 81173173). We sincerely thank Shanghai Sensichip Infotech Co. Ltd for their technical support.

References


