Research paper

Validation of aspirin response-related transcripts in patients with coronary artery disease and preliminary investigation on CMTM5 function


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ABSTRACT

Aspirin is widely used in the prevention of cardiovascular diseases, but the antiplatelet responses vary from one patient to another. To validate aspirin response related transcripts and illustrate their roles in predicting cardiovascular events, we have quantified the relative expression of 14 transcripts previously identified as related to high on-aspirin platelet reactivity (HAPR) in 223 patients with coronary artery disease (CAD) on regular aspirin treatment. All patients were followed up regularly for cardiovascular events (CVE). The mean age of our enrolled population was 75.80 ± 8.57 years. HAPR patients showed no significant differences in terms of co-morbidities and combined drugs. Besides, the relative expression of HLA-DQA1 was significantly lower in low on-aspirin platelet reactivity (LAPR) patients, when compared with HAPR and high normal (HN) group (p = 0.028). What's more, the number of arteries involved, HAPR status and the relative expression of CLU, CMTM5 and SPARC were independent risk factors for CVE during follow up (p < 0.05). In addition, overexpression of CMTM5 attenuated endothelial cells (ECs) migration and proliferation, with significantly decreased phosphorylated-Akt levels, while its inhibition promoted these processes in vitro (p < 0.05). Our study provides evidence that circulating transcripts might be potential biomarkers in predicting cardiovascular events. CMTM5 might exert anti-atherosclerotic effects via suppressing migration and proliferation in the vessel wall. Nevertheless, larger-scale and long-term studies are still needed.

1. Introduction

Coronary artery disease (CAD) has become the leading cause of mortality all over the world. As one of the most severe complications of atherosclerosis, intravascular thrombosis can trigger myocardial infarction and stroke. The activation and aggregation of platelets are known to play crucial roles in the atherothrombotic process (Viles-Gonzalez et al., 2004; Broos et al., 2012).

Aspirin, which exerts platelet inhibition effects by acetylating the serine residue in position S29 of cyclooxygenase (COX)-I and COX-2, thereby reducing the production of thromboxane A2 (TXA2), has been widely used in the secondary prevention of cardiovascular diseases (Pettersen et al., 2015). Nevertheless, antiplatelet responses to aspirin vary from one patient to another, severely limiting its protective effects in about 8–10% of patients on aspirin therapy.

High on-aspirin platelet reactivity (HAPR) is characterized as the phenomenon of insufficient inhibition of platelet aggregation during aspirin therapy. Various platelet function tests (PFTs) have been developed to evaluate platelet reactivity, and the link between HAPR and significantly increased risk of ischemic events, heart attack and
stroke has been well established in previous studies (Krasopoulou et al., 2008; Kuzniatsova et al., 2012; Steiner and Moertl, 2013; Oh et al., 2016). However, due to poor agreement among PFTs, there is still no consensus on how to define HAPR. The lack of correlation between PFTs and clinical outcomes also raises some concern (Cattaneo, 2013; Gurbel et al., 2016). Several explanations of HAPR have been proposed, including low compliance, declined absorption, simultaneous NSAIDs administration, high platelet turnover, non-platelet TXA2 synthesis during pathological conditions and others. Polymorphism of several genes, including P2RY1, P2RY12, COX-1, COX-2, GPIb and GPIIIa, were associated with HAPR (Timur et al., 2012; Yi et al., 2017). However, the genetic variability in HAPR has not been fully elucidated (Kuzniatsova et al., 2012).

Advanced age is a crucial risk factor for CAD and venous thromboembolism (VTE) in the general population, with increased mortality and worse prognosis. However, suboptimal platelet inhibition during treatment occurred more frequently in the elderly, which has been linked to higher risk of stroke and adverse events (Verdoia et al., 2016). Thus, developing circulating biomarkers in predicting HAPR might be beneficial in optimizing treatment strategies as well as avoiding adverse effects in the elderly, especially in patients aged > 75 years (Andreotti et al., 2015; Cuzick et al., 2015).

CMTM5, firstly identified by researchers in Peking University Center for Human Disease Genomics, is a novel member of human CKLF-like MARVEL transmembrane domain containing factor family, which includes at least six alternatively spliced forms, and CMTM5-v1 is the main form (Han et al., 2003). Previous studies revealed inhibitory effects of CMTM5 on proliferation, migration, and invasion of carcinoma cells through various signaling pathways (Li et al., 2010; Yuan et al., 2012). In our study, the correlation between CMTM5 expression with cardiovascular events, and the potential roles of CMTM5 on endothelial cells (ECs) migration and proliferation were investigated.

In recent years, genome-wide expression analysis has identified novel biomarkers and potential therapeutic targets for HAPR patients, which could lead to better antiplatelet regimens and decreased morbidity and mortality. Voora et al. screened out a set of co-expressed genes correlated with aspirin exposure in healthy volunteers (Voora et al., 2013). Fallahi et al. identified differentially expressed transcripts between patients with and without HAPR using microarray assay (Fallahi et al., 2013). However, evidence in Chinese patients was still lacking in this area.

Thus, the purpose of our study was to validate established aspirin response related transcripts in Chinese patients with CAD, and to investigate their potential roles in predicting cardiovascular events (CVE). The patients enrolled in this study were older than 75 years of age. In addition, we focus on the potential effects of CMTM5 on ECs proliferation and migration.

2. Methods and materials

2.1. Study design

The study included clinically stable patients of both genders with CAD, who were on regular aspirin therapy (100 mg/day). Inclusion criteria were as follows:

1. Presence of at least one of the following: stable angina pectoris (SAP); acute coronary syndrome (ACS); percutaneous coronary intervention (PCI); coronary artery bypass graft (CABG); confirmed coronary atherosclerotic plaques through computed tomographic angiography (CTA) or coronary arteriography (CAG) examinations
2. Age: older than 50 years
3. Volunteer for this study.

Patients were excluded when there existed contraindications for aspirin and co-administration of non-steroid anti-inflammatory drugs (NSAIDs), glycoprotein IIa/IIia inhibitors or warfarin. Patients with hematological diseases, severe liver or renal dysfunction, peptic ulcer or history of gastrointestinal hemorrhage were excluded.

The protocol was approved by the Ethical Review Committee of Peking University First Hospital, and written informed consent was acquired from all enrolled participants. All patients were followed up regularly in the outpatient department, with specific questions on cardiovascular symptoms, compliance and re-hospitalization for any reason. Cardiovascular event was defined as the occurrence of myocardial infarction, stroke, cardiovascular death and/or revascularization during regular aspirin therapy.

2.2. Light transmission assay

Light transmission aggregometry of arachidonic acid (AA, 0.5 mg/mL) induced platelet aggregation (LTA-AA) were measured in order to evaluate aspirin responses as described (Gum et al., 2003; Amsallem et al., 2015). Peripheral blood samples were drawn in a sodium citrate tube, and all measurements were carried out within 2 h. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP), used as reference, were prepared through centrifugation at separate speeds (200 × g 10 min for PRP; 2000 × g 10 min for PPP). AA-induced platelet aggregation was performed using a LBY-NJ4 platelet aggregometer (PRECl, Beijing). The percentage of platelet aggregation was defined as the maximal light transmittance after AA addition. In addition, adenosine diphosphate (ADP, 20 μmol/L) induced platelet aggregation (LTA-ADP) was also measured to exclude patients with poor response to thienopyridines.

2.3. Quantitative PCR analysis

Whole blood RNA was extracted using QIAamp Blood RNA Mini Kit (QIAGEN, Hilden, Germany). The concentration of RNA was assessed by absorbance at 260/280 nm using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Reverse transcription (RT) reactions were executed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA), in accordance to the manufacturer’s protocol. All cDNAs were stored at −80 °C for further quantitative analysis.

Quantitative PCR was performed using ABI 7500 quantitative PCR instrument in triplicates. All samples were normalized to 18S transcript levels, and their relative expression were determined using comparative threshold cycle method. The 20 μL reaction system consisted of 2 × SYBR® Select Master Mix (10 μL, Applied Biosystem, USA), sense/anti-sense primers (10 nM, 0.4 μL each), cDNA (1.6 μL) and ddH2O (7.6 μL). Melting curves and agarose gel electrophoresis were used to ensure mono-product amplification in each reaction.

2.4. Cell culture, transfection and reagents

To further illustrate potential mechanisms of CMTM5 on endothelial cell proliferation and migration, human umbilical vein endothelial cell line (EA.hy926) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) for in vitro experiments. Endothelial cells were maintained in Dulbecco modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (GibcoBRL, Life Technologies, Grand Island, NY, USA) in a humidified atmosphere of 5% CO2 atmosphere at 37 °C.

The construction and purification of adenovirus vectors (ad-CMTM5-v1 and ad-mock) were performed by ViGene Biosciences, Inc. (Rockville, USA), while RNA-interference lentivirus vectors were generated and purified in Beijing PreGene Biotechnology Company Ltd. (Beijing, PR China). Cells were infected as described, ad-pEGFP was used to monitor the efficiency of infection, and cells with > 75% infection efficiency were used for further study.
2.5. Cell proliferation assay

Groups of transfected cells were lifted with 0.25% trypsin and counted every 24 h. Cell proliferation was analyzed using the MTT Cell Proliferation Assay Kit (KeyGen Biotech, Nanjing, PR China) according to manufacturer’s instructions. 50 μL MTT reagent (2 mg/mL) were added to each well and incubated for 4 h, then DMSO (150 μL per well) was added after culture supernatant discarded. Cells were incubated in a shaker at 37 °C for 10 min until the crystals completely dissolved. The number of viable cells was calculated by absorbance measurements at 570 nm. Besides, in BrdU incorporation assay, cultured ECs were incubated with BrdU for 12 h before ethanol fixation. Incorporated BrdU was detected immunohistochemically. In addition, cell cycle analysis was carried out in serum-free medium for 24 h and then stimulated with medium containing 10% FBS for 20 h. After overnight ethanol immobilization and propidium iodide staining, all samples were analyzed on an FACS Calibrated flow cytometer (BD Biosciences), and the data were processed using FlowJo 9 software (FlowJo, Ashland, OR, USA).

2.6. Wound healing assay

Transfected cells were cultured in 6-well plate until confluent, cell mobility was evaluated using a wound healing assay. The cell layer was carefully wounded using sterile tips and washed twice with serum-free DMEM. After incubation for 0, 24, and 48 h, the cells were photographed at low magnification (40 ×) as described.

2.7. Cell migration assay

EA.hy926 cells, maintained in conditioned medium containing 0.5 mg/mL bovine serum albumin for 24 h, were added to the bottom of the 24-well chemotaxis chamber (Corning, NY, USA). A polycarbonate filter (8-μm pore size) was used. The transfected cells were trypsinized and washed twice, and 50 μL of the single-cell suspension (0.5 × 10^5 cells/mL in 0.1% bovine serum albumin/DMEM) was added to the upper well of the chamber. After incubation for 12 h at 37 °C in a 5% CO₂ humidified atmosphere, non-migrated cells in the upper chamber were scraped off and the migrated cells on the bottom of the filter were fixed with methanol and stained with crystal violet. Cells that migrated through the filters were counted from at least six randomly selected fields in each experiment. Results were obtained from at least three individual experiments and represented as the cell migration index, which was the number of cells per high power field.

2.8. Western blot

Cell lysates were extracted with the RIPA lysis buffer (KeyGen Biotech, Nanjing, PR China) with a cocktail of phosphates inhibitors (Cell Signaling Technology, Denver, MA, USA). Equal amounts of total protein (20 μg) were separated by SDS-PAGE and transferred onto nitrocellulose membrane (Millipore). After blocking for non-specific binding, the membranes were incubated with anti-PI3K antibody, anti-Akt antibody, anti-pAkt antibody (1:1000 dilution; Cell Signaling Technology), anti-CMTM5 antibody or anti-GAPDH antibody (Transgen Biotech, Beijing, PR China) overnight at 4 °C followed by HRP-conjugated secondary antibodies for 1 h at room temperature. After washing three times in TBST, protein bands were visualized using chemiluminescence method. The polyclonal antibody against CMTM5 of human origin (epitope mapping at the C-terminus, capable of detecting CMTM5 isoforms 1–5 of human origin) was given as gifts by Prof. Wenling Han (Peking University Center for Human Disease Genomics, Beijing, China).

2.9. Statistical analysis

Continuous variables were expressed as mean ± standard deviation, while categorical variables were described as frequency and percentage. Conformity to normal distribution was evaluated for continuous variables using both Kolmogorov-Smirnov and Shapiro-Wilk tests. The Student t-test or Nonparametric Mann-Whitney U test were used to make comparisons for continuous variables, while Chi-square or Fisher exact-test were applied for categorical variables. COX regression analysis was performed to investigate potential risk factors for CVE. A 2-tailed p-value < 0.05 was considered as statistically significant for all analysis executed. Statistical analysis was carried out using SPSS version 16.0 software (SPSS Inc., Chicago, Illinois).

3. Results

We have quantified the relative expression of 14 transcripts previously identified as related to HAPR in 223 CAD patients on regular aspirin treatment. Using LTA-AA quartiles for grouping, the relative expression of HLA-DQA1 was significantly lower in low on-aspirin platelet reactivity (LAPR) patients. What’s more, the number of arteries involved, HAPR status and the relative expression ofCLU, CMTM5 and SPARC were independent risk factors for CVE during follow up (p < 0.05). In addition, we focused on the potential anti-atherosclerotic effects of CMTM5 via suppressing ECs migration and proliferation.

3.1. Enrollment

A total of 315 patients were enrolled since January 2014. From enrolled patients, 30 were excluded for non-compliance with aspirin treatment regimen and 3 because of severe renal dysfunction (eGFR < 15 mL/min). In addition, 35 patients were excluded because of the absence of atherosclerotic plaques on CTA or CAG examinations. 24 patients were excluded for the absence of LTA-AA detection. The remaining 223 patients with coronary artery disease who satisfied the inclusion criteria were included for further analysis (Supplementary Fig. S1).

3.2. Light transmission assay

Arachidonic acid-induced platelet aggregation (LTA-AA) was measured in the 223 patients, and the quartiles were 9.48%, 11.79% and 14.75%, respectively. In our enrolled patients, HAPR was defined as LTA-AA greater than the upper quartile (LTA-AA > 14.75%), while Non-HAPR group was further divided into two parts: low on-aspirin platelet reactivity (LAPR), defined as LTA-AA less than or equal to the lower quartile (LTA-AA ≤ 9.48%, n = 56); High normal (HN), defined as LTA-AA ranged from 9.48% to 14.75% (n = 112).

When compared with patients aged 50 to 75 years, those older than 75 years had higher platelet reactivity during regular aspirin treatment (mean LTA-AA value: 13.18 ± 4.74% versus 11.98 ± 4.19%, p = 0.038) (Fig. 1A), while the incidence of HAPR was not significantly higher in the > 75 years group (28.46% versus 18.60%, p = 0.096) (Fig. 1B).

In patients received dual antiplatelet therapy, none was with low clopidogrel response status according to LTA-ADP. The Distribution of LTA-AA and LTA-ADP in patients receiving DAPT were shown in Fig. 1C.

3.3. Baseline clinical features

Comparisons were made between patients with and without HAPR in terms of clinical features, combined drugs and laboratory characteristics. The mean age of our enrolled population was 75.80 ± 8.57 years, and patients older than 60 years accounted for nearly 95% (212 of 223). Female patients were 18.39% of the overall
Co-morbidities, combined drugs, and laboratory characteristics are shown in Table 1. When compared with patients without HAPR, no significant differences in terms of co-morbidities and combined drugs were observed in HAPR patients. However, HAPR patients exhibited a tendency to be older (p = 0.021) and with worse renal function (p = 0.099). In addition, platelet count (PLT) and hematocrit (Hct) were significantly lower in HAPR patients (p < 0.05) (Table 1).

3.4. Relative expression of fourteen transcripts

Fourteen transcripts were selected for quantitative PCR analysis, including CLU, CTTN, CMTM5, MPL, TMEM64, HLA-DQA1, HLA-DRB4, SELP, ITGA2B, ITGB3, THBS1, SPARC, PPBP and CXCL5, which showed correlation with aspirin response in previous US studies (Fallahi et al., 2013; Voora et al., 2013). Seven transcripts were overlapped in two US studies, including CLU, CTTN, CXCL5, ITGA2B, ITGB3, MPL and THBS1. What's more, we also selected transcripts with remarkable fold changes and with lower p values. Primers used in quantitative PCR reactions are listed in Supplementary Table S1.

In our enrolled population, the relative expression of HLA-DQA1 was significantly lower in HAPR patients, when compared with HN and HAPR group (p = 0.028) (Fig. 2), and the potential mechanisms warrant further study. Spearman’s correlation analysis confirmed an inverse correlation between HLA-DQA1 and LTA-AA (r = −0.142, p = 0.05). Comparison with Fallahi et al. study on gene expression was shown in Supplementary Table S2.

3.5. Potential risk factors for CVE

All patients were followed up in the outpatient department regularly, with specific questions about cardiovascular symptoms, compliance, re-hospitalization for any reasons, and other events. The median follow up of our enrolled patients was 18 months (range 3 to 22 months), and no one dropped out during follow-up. 40 patients (17.9%) experienced cardiovascular events during our follow up, including 6 non-fatal stroke, 7 readmission for acute coronary syndrome (ACS), and 27 receiving revascularization.

Potential risk factors for CVE were investigated using COX regression analysis. Hazard ratio (HR) was calculated, and p value < 0.05 was defined as statistically significant. As shown in Table 2, each additional artery involved was associated with 2.320 times higher risk for CVE (HR 2.320, 95%CI 1.397–3.854, p = 0.001). HAPR patients were at an almost 2.8 times higher risk in developing CVE (HR2.778, 95%CI 1.188–6.497, p = 0.018). Furthermore, the relative expression of CLU, CMTM5 and SPARC were independent risk factors for CVE (p < 0.05) (Table 2). We also made comparison between our study and Voora et al. study on the hazard ratio(HR) of these transcripts (Supplementary Table S3).

3.6. Overexpression of CMTM5-v1 inhibits ECs migration in vitro

CMTM5, a novel member of human CKLF-like MARVEL transmembrane domain containing factor family, was firstly identified by researchers in Peking University Center for Human Disease Genomics (Han et al., 2003). Although previous studies focused on the inhibitory effects of CMTM5 on tumor cell proliferation, migration and invasion (Li et al., 2010), our study revealed that CMTM5 expression profile was an independent risk factor for cardiovascular events. To further illustrate CMTM5 potential roles in the setting of cardiovascular diseases, we used CMTM5 overexpression and RNA-interference vectors and investigate its effect on endothelial cell migration and proliferation.

To evaluate the roles of CMTM5-v1 on cell migration, a wound healing assay, and a transwell migration model were used to assess cell's horizontal and vertical mobility. As shown in Fig. 3A, overexpression of CMTM5 inhibited ECs migration. We also examined the motility of transfected cells using a Boyden chamber migration assay. As shown in Fig. 3B, the migration of ad-CMTM5-v1-transfected cells...
<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Non-HAPR</th>
<th>HN</th>
<th>HAPR</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LAPR (n = 56)</td>
<td>HN (n = 112)</td>
<td>HAPR (n = 55)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>73.68 ± 9.62</td>
<td>75.71 ± 8.60</td>
<td>78.90 ± 6.49</td>
<td>0.021</td>
</tr>
<tr>
<td>Female (%)</td>
<td>15 (26.78)</td>
<td>15 (13.39)</td>
<td>11 (20.00)</td>
<td>0.106</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.13 ± 3.74</td>
<td>24.74 ± 2.89</td>
<td>24.41 ± 2.84</td>
<td>0.453</td>
</tr>
<tr>
<td>Previous PCI (%)</td>
<td>33 (58.92)</td>
<td>65 (58.03)</td>
<td>33 (60.00)</td>
<td>0.971</td>
</tr>
<tr>
<td>Smoking history (%)</td>
<td>24 (42.85)</td>
<td>47 (41.96)</td>
<td>21 (41.18)</td>
<td>0.876</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>38 (67.85)</td>
<td>87 (77.68)</td>
<td>41 (74.54)</td>
<td>0.388</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>20 (35.71)</td>
<td>41 (36.61)</td>
<td>24 (43.64)</td>
<td>0.620</td>
</tr>
<tr>
<td>Hyperlipidemia (%)</td>
<td>10 (17.86)</td>
<td>36 (32.14)</td>
<td>20 (36.36)</td>
<td>0.061</td>
</tr>
<tr>
<td>Ischemia cerebrovascular disease (%)</td>
<td>42 (75.00)</td>
<td>92 (82.14)</td>
<td>48 (87.27)</td>
<td>0.243</td>
</tr>
<tr>
<td>Combined drugs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dual antiplatelets (%)</td>
<td>26 (46.42)</td>
<td>53 (47.32)</td>
<td>32 (58.19)</td>
<td>0.244</td>
</tr>
<tr>
<td>Statins (%)</td>
<td>56 (100.00)</td>
<td>105 (93.75)</td>
<td>53 (96.36)</td>
<td>0.150</td>
</tr>
<tr>
<td>ACEI (%)</td>
<td>17 (30.35)</td>
<td>23 (20.53)</td>
<td>12 (21.82)</td>
<td>0.369</td>
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<tr>
<td>ARB (%)</td>
<td>15 (26.78)</td>
<td>34 (30.36)</td>
<td>21 (41.18)</td>
<td>0.410</td>
</tr>
<tr>
<td>β blockers (%)</td>
<td>43 (76.78)</td>
<td>83 (74.11)</td>
<td>40 (72.72)</td>
<td>0.881</td>
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<tr>
<td>Anti-diabetes (%)</td>
<td>16 (28.57)</td>
<td>35 (31.25)</td>
<td>18 (32.73)</td>
<td>0.889</td>
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<tr>
<td>Nitrates (%)</td>
<td>20 (35.71)</td>
<td>50 (44.64)</td>
<td>24 (43.64)</td>
<td>0.502</td>
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<tr>
<td>CCB (%)</td>
<td>20 (35.71)</td>
<td>51 (45.53)</td>
<td>25 (45.45)</td>
<td>0.436</td>
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<tr>
<td>PPI (%)</td>
<td>13 (23.21)</td>
<td>36 (32.14)</td>
<td>20 (36.36)</td>
<td>0.302</td>
</tr>
<tr>
<td>H2R antagonist (%)</td>
<td>8 (14.29)</td>
<td>11 (9.82)</td>
<td>10 (18.18)</td>
<td>0.310</td>
</tr>
<tr>
<td>Biological characteristics</td>
<td></td>
<td></td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>eGFR (mL/min)</td>
<td>65.49 ± 17.20</td>
<td>64.99 ± 15.29</td>
<td>59.7 ± 17.20</td>
<td>0.099</td>
</tr>
<tr>
<td>25-OH-D (pg/mL)</td>
<td>37.14 ± 12.81</td>
<td>40.57 ± 16.89</td>
<td>42.06 ± 24.52</td>
<td>0.339</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.00 (5.10–8.30)</td>
<td>5.95 (5.10–9.80)</td>
<td>6.00 (5.20–9.00)</td>
<td>0.821</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>60.0 (32.0–70.0)</td>
<td>60.0 (32.4–79.6)</td>
<td>60.7 (43.0–70.1)</td>
<td>0.521</td>
</tr>
<tr>
<td>Hcy (µmol/L)</td>
<td>13.62 ± 3.56</td>
<td>13.98 ± 8.11</td>
<td>13.13 ± 4.03</td>
<td>0.604</td>
</tr>
<tr>
<td>PLT (×10⁹/L)</td>
<td>195.11 ± 82.92</td>
<td>193.07 ± 53.91</td>
<td>166.94 ± 52.18</td>
<td>0.016</td>
</tr>
<tr>
<td>MPV (fl)</td>
<td>8.50 ± 1.28</td>
<td>8.43 ± 1.00</td>
<td>8.49 ± 1.14</td>
<td>0.915</td>
</tr>
<tr>
<td>Hb (g/L)</td>
<td>132.43 ± 17.18</td>
<td>132.39 ± 20.45</td>
<td>128.00 ± 14.43</td>
<td>0.126</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>37.92 ± 4.68</td>
<td>38.11 ± 4.83</td>
<td>36.20 ± 4.09</td>
<td>0.022</td>
</tr>
</tbody>
</table>

Values are mean ± SD or median (range).

HAPR indicates high on-aspirin platelet reactivity; LAPR, low on-aspirin platelet reactivity; HN, high normal; BMI, body mass index; PCI, percutaneous intervention; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor antagonist; CCB, calcium channel blockers; PPI, proton pump inhibitors; LVEF, left ventricular ejection fraction; Hcy, homocysteine; PLT, platelet count; MPV, mean platelet volume; Hb, hemoglobin; Hct, hematocrit.

* p < 0.05.
was remarkably suppressed, while that of RNA-interference lentivirus infected cells was promoted, when compared with mock-transfected cells, indicating that *CMTM5* strongly inhibits the migration of human umbilical vein endothelial cells (HUVECs).

### 3.7. CMTM5 suppression facilitates the proliferation of ECs in vitro

Cell counting and MTT assays revealed that overexpression of *CMTM5* induced time-dependent inhibition of ECs proliferation (Fig. 4A and B), while inhibition of *CMTM5* expression facilitated their growth (Fig. 5A and B). Moreover, BrdU incorporation was used to confirm the proliferation-promoting effect of *CMTM5* suppression. Percentage of BrdU-positive cells was significantly higher in ES group, which was indicated in the red box (Fig. 5C and D). In addition, the percentage of S + G2 phase cells was quantified using flow cytometry assay (FCM). It was observed that the percentage of S + G2 phase cells in EO group was 13.55%, significantly lower than that in EN (21.43%) and EO-mock group (23.24%) \((p < 0.05, \text{Fig. 4C} \text{ and D})\). What’s more, the percentage of S + G2 phase cells in ES group was 40.98%, significantly higher than that in EN (20.53%) and ES-mock group (22.82%) \((p < 0.05, \text{Fig. 5E} \text{ and F})\).

### 3.8. The effect of CMTM5 levels on the PI3K and Akt pathways

To elucidate the molecular mechanisms underlying ECs proliferation and migration inhibition by overexpression of *CMTM5*, several proteins possibly involved in this process were detected using western blot. It was observed that protein levels of p-Akt in EO group were significantly decreased, when compared with ES and EN groups, while no significant differences were observed in PI3K and Akt protein levels (Fig. 6). This suggests that *CMTM5* may repress ECs proliferation and migration through regulating PI3K-Akt signaling, one of the most important pathways involved in cell development.

### 4. Discussion

Although several studies have been performed to identify genes associated with platelet reactivity after aspirin therapy, evidence in Chinese patients was lacking in this area. The patients enrolled in this study were older than previous studies. In our study, we choose 14 transcripts showed obvious correlation with aspirin response in previous US studies, and revealed relative expression of several transcripts as potential cardiovascular risk factors in CAD patients. Lower relative expression was observed in LAPR patients, when compared with HAPR patients. Further, transcripts including *CLU*, *CMTM5*, and *SPARC* were independent risk factors for CVE. However, our results did not agree with some aspects of previous US studies, which may be due to obvious differences in ethnic/genetic factors, patient age, gender ratio, duration of follow-up, platelet reactivity methods, mRNA biomarker quantization methods, and other unknown factors.

**Table 2**

<table>
<thead>
<tr>
<th>CV risk factors</th>
<th>Hazard ratio(HR)</th>
<th>95%CI lower</th>
<th>95%CI upper</th>
<th>(p) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arteries involved</td>
<td>2.320</td>
<td>1.397</td>
<td>3.854</td>
<td>0.001(^*)</td>
</tr>
<tr>
<td>(\Delta C_{LU})</td>
<td>1.386</td>
<td>1.185</td>
<td>1.621</td>
<td>&lt; 0.001(^*)</td>
</tr>
<tr>
<td>(\Delta C_{CMTM5})</td>
<td>0.831</td>
<td>0.705</td>
<td>0.978</td>
<td>0.026</td>
</tr>
<tr>
<td>(\Delta C_{SPARC})</td>
<td>0.804</td>
<td>0.693</td>
<td>0.932</td>
<td>0.004</td>
</tr>
<tr>
<td>HAPR</td>
<td>2.778</td>
<td>1.188</td>
<td>6.497</td>
<td>0.018</td>
</tr>
</tbody>
</table>

\(^*\) \(p < 0.05\).

\(^*\) \(p < 0.01\).

\(^*\) \(p < 0.001\).

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**Fig. 3.** Effects of CMTM5 on ECs migration.

Wound healing assay and transwell migration model were used to assess CMTM5 effects on ECs migration (horizontal and vertical mobility).

(A) Wound healing assay: ad-CMTM5-v1 and RNA-interference lentivirus infected cells were incubated for 48 h after wounded. Overexpression of CMTM5 inhibited ECs migration.

(B) Boyden chamber migration assay: the migration of ad-CMTM5-v1-transfected cells was remarkably suppressed, while that of RNA-interference lentivirus infected cells was promoted, when compared with mock-transfected cells.
aspirin exposure were identified, including CLU, CMTM5, CXCL5, and MPL. Association of these transcripts with death/MI were validated in two patient cohorts (Voora et al., 2013). Fallahi et al. identified differentially expressed transcripts between patients with and without HAPR using microarray assay, including HLA-DQA1, HLA-DRB4, and several platelet transcripts such as clusterin (CLU), glycoproteins IIb/IIIa (ITGA2B/3), lipocalin (LCN2), and lactoferrin (LTF). Differently, obvious lower expression of HLA-DQA1 was observed in aspirin resistant patients (Fallahi et al., 2013). In Zufferey et al. (2016) study, two miRNAs (miR-135a-5p and miR-204-5p) and their predicted targets including THBS1, CDC42, CORO1C, SPTBN1, TPM3, GTPBP2, and MAPRE2 were demonstrated to be different in patients with varied platelet reactivity. Furthermore, Nagalla et al. reported 3 miR-mRNA pairs related to platelet activation via genome-wide profiling in 19 healthy subjects, including miR-200b: PRKAR2B, miR-495:KLHL5 and miR-107:CLOCK (Nagalla et al., 2011).

HLA-DQA1, differentially expressed in LAPR patients, is strongly associated with several autoimmune diseases, including Type 1 Diabetes (T1DM), systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (Zhao et al., 2016). Recently, a weak link between HLA-DQA1 genotype and acute myocardial infarction was demonstrated (Bjorkbacka et al., 2010). As independent risk factors for CVE, CLU and SPARC were among the top 50 abundant transcripts in human platelets (Gnatenko et al., 2003). CLU, involved in numerous physiological processes, exhibits cytoprotective and anti-inflammatory effects via interacting with known inflammatory proteins (Yang and Qin, 2015). Lowered circulating CLU levels was reported to be involved with thrombotic complications in SLE patients (Wang et al., 2004). SPARC plays a role in regulating fibroblast migration and cell-extracellular matrix interactions. It was revealed that platelet-secreted glycoprotein SPARC was down-regulated in patients with acute coronary syndrome (ACS) (Parguina et al., 2010). Furthermore, SPARC derived from adipose tissue was associated with insulin resistance and diabetes complications (Kos and Wilding, 2010).

Previous studies revealed inhibitory effects of CMTM5 on proliferation, migration, and invasion of carcinoma cells through various signaling pathways (Li et al., 2010; Yuan et al., 2012). In our study, the profiles of CMTM5 expression was an independent risk factor for cardiovascular events in our enrolled patients. Similarly, in Voora et al. study, the increased profiles of CMTM5 gene expression was associated with the increased risk of MI and mortality (Voora et al., 2013). Thus, the potential mechanisms of CMTM5 in coronary artery disease warrants further study. For this reason, we evaluated the effects of CMTM5 overexpression or suppression on ECs migration and proliferation in vitro. In our study, inhibition of CMTM5 expression promoted proliferation and migration of ECs, which might indicate the crucial role of CMTM5 in endothelial integrity and repair after vascular injury.

Our study demonstrated that HAPR was associated with almost 28 times higher risk for CVE. No consensus on HAPR cut-offs has been proposed, partly due to the various kinds of PFTs performed in these studies, as well as the poor agreements observed (Gremmel et al., 2011; Blann et al., 2013). As a result, although numerous studies have been performed to link HAPR with adverse clinical outcomes, disagreements among studies were reported. In Nagatsu et al.’s multicenter study, a negative association was observed between HAPR and CVE after two-year follow up in stable cardiovascular outpatients (Nagatsu et al., 2016). Campo et al. observed that aspirin response evaluation failed to predict worse outcomes in patients receiving percutaneous coronary interventions (PCI) (Campo et al., 2010). Furthermore, in D’Ascenzo et al. meta-analysis, HAPR did not significantly increase the risk of cardiac event in ACS patients or patients with stable angina (D’Ascenzo et al., 2014). However, in Wisman et al.’s meta-analysis, HAPR was
associated with an increased CVE risk (RR 2.09, 95%CI 1.77–2.47, \( p < 0.05 \)) (Wisman et al., 2014).

As mentioned, the elderly accounted for nearly 95% of our overall patients (212 of 223). The elderly accounted for a considerable proportion of cardiovascular patients at higher risk of both ischemic and bleeding risks. Besides, poorer antiplatelet responses were observed in this particular population. However, the included proportion of the elderly in large-scale randomized controlled trials (RCTs) were rather low, thus making it difficult to optimize antiplatelet treatment strategies (Andreotti et al., 2015; Cuzick et al., 2015). Detecting circulating biomarkers might be beneficial in evaluating HAPR status, as well as predicting cardiovascular risks.

Our studies have several unique strengths. To our knowledge, this is the first study to investigate aspirin response related genes in Chinese patients, and several transcripts have been identified as independent risk factors for CVE. Our results have the greatest relevance for the elderly Chinese population at risk of cardiovascular diseases. Furthermore, our studies suggest that the effects of CMTM5 on ECs migration and proliferation deserves further study. Nevertheless, there are several limitations in our studies. Since it is a single-center study, these results may not be extrapolated to all Chinese patients with cardiovascular diseases. Larger-scale and long-term studies are still needed to attain consensus on HAPR definition, and to elucidate potential roles of these transcripts in cardiovascular diseases.

To conclude, our study provides further evidence that circulating transcripts might become potential biomarkers in predicting cardiovascular events. However, larger-scale and long-term studies are still needed. Also, it was observed that CMTM5 might play an important role in ECs migration and proliferation. This may provide a promising therapeutic target for the prevention and treatment of coronary artery disease.

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**Disclosure**

The authors do not report any relevant conflicts of interest.

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Fig. 6. The effect of CMTM5 levels on the PI3K and Akt pathway proteins.
To elucidate the molecular mechanisms underlying ECs proliferation and migration inhibition by overexpression of CMTM5, protein levels of PI3K, Akt and phosphorylated-Akt (p-Akt) were detected using western blot.

(A–B) The protein levels of p-Akt in ES group was significantly increased, when compared with EN and ES-MOCK groups (p < 0.05).

(C–D) The protein levels of p-Akt in EO group were significantly lower than that in EN and EO-MOCK groups (p < 0.05).

also thank Prof. Wenling Han (Peking University Center for Human Disease Genomics) for giving us anti-CMTM5 polyclonal antibody and pCDB-CMTM5-v1 plasmid as kind gifts.

Appendix A. Supplementary data
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2017.04.041.

References


