Prevalence of baseline NS3 resistance-associated substitutions (RASs) on treatment with protease inhibitors in patients infected with HCV genotype 1

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KEYWORDS
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Summary
Background and aims: Treatment for hepatitis C has evolved significantly with the licensing of direct-acting antiviral drugs (DAAs). However, one of the limiting factors of the effectiveness of antiviral therapy with protease inhibitors (PIs) is the emergence of resistance caused by point mutations. The aim of this study was to determine the prevalence of resistance-associated substitutions (RASs) in HCV NS3 gene in patients infected with genotype 1 before therapy with simeprevir.

Methods: A total of 73 serum samples from 15 treatment-experienced patients with boceprevir/telaprevir and 58 DAA-naïve patients were collected before therapy with DAAs simeprevir, daclatasvir and/or sofosbuvir. Presence of baseline resistance-associated substitutions (RAS) in the serine protease domain of HCV NS3 was analyzed by nucleotide sequencing followed by amino acid deduction.

Results: Overall RAS prevalence in this study was 13.7% (10/73). RAS prevalence for HCV subtype 1b was 17.4% (4/23) while for HCV subtype 1a was 12% (6/50). Primary mutations V36M/L and *Corresponding author at: Laboratório de Hepatites Virais, Instituto Oswaldo Cruz, FIOCRUZ, avenida Brasil, 4365 — Manguinhos, Rio de Janeiro, RJ, Brazil. E-mail address: v.duarte391@gmail.com (V.D. Costa).

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Introduction

It is estimated that 71 million people worldwide are chronically infected with hepatitis C virus (HCV) and the number of deaths each year mostly from cirrhosis and hepatocellular carcinoma is approximately 399,000 [1]. An important clinical aspect of hepatitis C is the high rate of progression to chronicity observed in about 85% of individuals infected by HCV. A substantial fraction of these chronic carriers might develop progressive liver fibrosis, eventually leading to cirrhosis and hepatocellular carcinoma (HCC) [2]. HCV is classified in seven genotypes (1–7) and 67 subtypes [3]. The most common subtypes in Western countries are 1a and 1b [4].

Until 2011, the therapy for chronic hepatitis C was based in a combination of pegylated-interferon and ribavirin (peg-IFN/RBV), a long-term therapy that, besides the severe undesirable side effects [5], had not produced encouraging results in sustained virological response (SVR) mainly for patients infected with HCV genotype 1 [6]. Due to this unsatisfactory therapeutic approach, a regimen with fewer side effects, reduced rates of patient withdrawal and increased effectiveness in preventing the progression to decompensated cirrhosis and HCC has been the focus of several studies of drug development and clinical trials during the last decade. Advances in cell cultures lineages permissive to HCV infection had represented a milestone in understanding about viral life cycle. Along with the three-dimensional computational modelling of viral proteins, several molecules capable of a specific inhibition of proteins acting in different stages of virus replication have been developed and nominated as direct-acting antiviral drugs (DAAs) [7]. Among the targets for DAA is the HCV NS3 serine protease, a protein that forms a non-covalent complex with NS4A and is responsible for the cleavage of the non-structural portion of the translated viral polyprotein.

In 2013, first-wave, first-generation drugs telaprevir and boceprevir was the first protease inhibitors (PIs) incorporated in Brazilian Clinical Guidelines for the treatment of patients infected with HCV genotype 1. Based on this 2013 Clinical Guideline, telaprevir could be used for both naïve and experienced patients whereas boceprevir was only indicated for treatment-naïve patients with advanced fibrosis METAVIR F3 and F4. Its combination with peg-IFN/RBV yielded an improvement in the SVR rate up to 75% [8–12]. Nonetheless, significant side effects and unsatisfactory efficiency against genotype 1 highlighted the necessity of development of compounds targeting different viral proteins in order to achieve higher SVR rates and viral clearance. More recently, other HCV PIs have been incorporated to Clinical Guidelines and can be prescribed irrespective of patients’ treatment records. Simeprevir, a second-wave, first-generation NS3/4A PI, daclatasvir (NS5A inhibitor) and sofosbuvir (NS5B polymerase inhibitor) were approved for clinical use in Brazil in 2015. Its genotypic coverage is broader than that of the first-wave drugs, including at least genotypes 1, 2, and 4 [13]. In 2017, paritaprevir in combination with ombitasvir, ritonavir and dasabuvir has been licensed in Brazil for subtype 1a patients without cirrhosis and for subtype 1b patients with compensated cirrhosis (Child–Pugh A). A second-generation PI, grazoprevir, co-administrated with elbasvir was incorporated in clinical protocol for patients infected with HCV genotype 1 and 4. Pangenotypic PIs, such as glecaprevir and voxilaprevir, are not yet registered in Brazilian regulatory agency however represents a promising alternative for patients with or without cirrhosis.

The occurrence of naturally HCV NS3 resistance-associated substitutions (RASs) affects virological outcome of DAA-based combination therapies [14–19]. For the majority of NS3 protease inhibitors the frequency of natural occurrence of single RASs in HCV genotype 1-infected patients is between 0.1% and 3.1% [20] and patients who failed to respond to simeprevir treatment had mutations at NS3 positions 80, 122, 155, and/or 168 [21]. Naturally occurring resistance have been reported in 4.1% to 18.9% of HCV infected patients with baseline NS3 mutations [22,23]. Detecting resistant variants at baseline in treatment-naïve patients infected with genotype 1 strains could represent an important background information to a more specific and efficient clinical conduct. The aim of this study was to determine the prevalence of naturally occurring RASs in the serine protease domain of HCV NS3 region in patients chronically infected with subtypes 1a and 1b.

R155K were observed only in HCV subtype 1a, whereas T54S and Q80K were identified only in HCV subtype 1b. RAS V36M, which is related to reduction of susceptibility to second-generation PIs, was the most frequent in the study (6.9%; 5/73).

Conclusions: Our results indicated that Brazilian isolates of HCV present a distinct pattern of RAS depending on the infecting viral subtype. In contrast to data from other countries, RAS Q80K prevalence in Brazil is low in HCV subtype 1a. This study improves the knowledge of genetic barrier for resistance to PIs involving RASs in chronically infected patients and its possible impact on an unsuccessful treatment outcome, information that might be crucial to upcoming decisions of incorporation of new DAAs in Brazilian guidelines of antiviral therapy against HCV infection.
Patients and methods

Study population

This study enrolled 73 individuals chronically infected with HCV genotype 1 who attended the Ambulatory of Viral Hepatitis (Oswaldo Cruz Institute) and Gaffrée & Guinile University Hospital between 2013 and 2016. Of the 73 individuals, 15 (1a: 14; 1b: 1) were treatment-experienced with first-wave, first-generation PIIs boceprevir/telaprevir (group 1) and 58 (1a: 36; 1b: 22) have not been experienced with DAAs (group 2) (Table 1). Serum samples were collected before therapy with DAAs, simeprevir, daclatasvir and/or sofosbuvir approved in 2015. The study included patients over 18 years old, both female and male, with positive diagnostic for chronic hepatitis C (Anti-HCV reagent for more than six months and confirmation with detectable HCV-RNA) and infected with HCV genotype 1 (subtypes 1a and 1b).

Ethical approval

Written informed consent was obtained from each patient before entering the study. This study was approved by the ethics committee from Oswaldo Cruz Foundation under number 142/01, and by the ethics committee of Gaffrée & Guinile University Hospital under number 204.445.

RNA extraction

RNA extraction from serum samples (200 μL) was done using High Pure Viral Nucleic Acid Kit (Roche Life Science, Mannheim, Germany) following manufacturer’s instructions. Extracted RNA was eluted in 50 μL and stored at −70 °C until further analysis.

Reverse-transcription and PCR amplification

Partial NS3 region of HCV genome covering nucleotides 3465−3961 (~500 bp) was amplified by one-step reverse-transcription (RT) with polymerase chain reaction (PCR) followed by a second round of PCR (Nested-PCR) using specific primers for each subtype (Table 2). The first round PCR amplification was carried out using reagents from the Superscript™ III One Step RT-PCR system (ThermoFisher, Massachusetts, USA). RT-PCR mixture contained 10 μM of specific sense and antisense primers, 2X reaction mix, SuperScript™ III RT/Platinum® Taq DNA Polymerase (4U/μL), RNaseOUT Recombinant Ribonuclease Inhibitor (ThermoFisher) and 5 μL of viral RNA. The conditions for RT-PCR were as follows: 45 °C for 30 min for reverse-transcription followed by an initial activation of DNA polymerase at 94 °C for 2 min, then 40 cycles at 94 °C for 15 sec, 60 °C for 30 sec and 68 °C for 120 sec and a final elongation at 68 °C for 5 min. Five microliters of the product was subjected to a second round of PCR that contained 10 μM of specific sense and antisense primers, 10× PCR buffer, 10 mM of dNTP, 50 mM of MgSO4 and Platinum® Taq High Fidelity (5U/μL). After an initial denaturation at 94 °C for 2 min, DNA was amplified for 30 cycles at 94 °C for 15 sec, 54 °C for 30 sec and 68 °C for 120 seconds. PCR products of the expected length of 495 and 496 base pairs for subtypes 1a and 1b, respectively, were fractionated by 1.5% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Patients characteristics according to HCV subtype.</th>
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<tr>
<td>Characteristics</td>
<td>Subtype</td>
</tr>
<tr>
<td>Gender</td>
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<tr>
<td>Male</td>
<td></td>
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<tr>
<td>Female</td>
<td></td>
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<tr>
<td>DAA-experienced patients (group 1) (telaprevir/boceprevir)</td>
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<td>DAA-naïve patients (group 2)</td>
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<td>Mean HCV viral load (IU/mL log10) ± SD</td>
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<th>Table 2</th>
<th>Primers for amplification of NS3 region of HCV subtypes 1a and 1b.</th>
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<tbody>
<tr>
<td>Primers</td>
<td>Polarity</td>
</tr>
<tr>
<td>1aF1</td>
<td>Sense</td>
</tr>
<tr>
<td>1aR1</td>
<td>Antisense</td>
</tr>
<tr>
<td>1aF2</td>
<td>Sense</td>
</tr>
<tr>
<td>1aR2</td>
<td>Antisense</td>
</tr>
<tr>
<td>1bF1</td>
<td>Sense</td>
</tr>
<tr>
<td>1bR1</td>
<td>Antisense</td>
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<tr>
<td>1bR2</td>
<td>Antisense</td>
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</tbody>
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Table 3 Amino acid mutations in the NS3 protein and their association to protease inhibitors used in clinical therapy for subtypes 1a and 1b of HCV.

<table>
<thead>
<tr>
<th>Amino acid position</th>
<th>RAS</th>
<th>Subtype 1a (n = 50)</th>
<th>Subtype 1b (n = 23)</th>
<th>Drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>V36</td>
<td>V36L</td>
<td>1</td>
<td>—</td>
<td>RS/RS</td>
</tr>
<tr>
<td></td>
<td>V36M</td>
<td>4</td>
<td>—</td>
<td>R/RS</td>
</tr>
<tr>
<td>F43</td>
<td>F43V</td>
<td>—</td>
<td>1</td>
<td>S/S</td>
</tr>
<tr>
<td>T54</td>
<td>T54S</td>
<td>—</td>
<td>1</td>
<td>RS/RS</td>
</tr>
<tr>
<td>Q80</td>
<td>Q80K</td>
<td>—</td>
<td>1</td>
<td>S/S</td>
</tr>
<tr>
<td></td>
<td>Q80H</td>
<td>—</td>
<td>1</td>
<td>S/S</td>
</tr>
<tr>
<td>V36+R155</td>
<td>V36M+R155K</td>
<td>1</td>
<td>—</td>
<td>R/R</td>
</tr>
</tbody>
</table>

S: susceptible; R: resistant; RS: reduced susceptibility (association to resistance, insufficient evidence for clinical outcome); TVR: telaprevir; BOC: boceprevir; SMP: simeprevir; PTV: paritaprevir; ASU: asunaprevir; GZV: grazoprevir.

Nucleotide sequencing

The NS3 nested-PCR products were purified using High Pure PCR Product Purification Kit (Roche Life Science) and DNA concentration of each sample was estimated with Low DNA Mass Ladder (ThermoFisher). Purified products were subjected to nucleotide sequencing reactions in both directions using the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions and analyzed on an ABI 3730 DNA automated sequencer (Applied Biosystems).

Mutation analysis

The obtained nucleotide sequences were assembled in MEGA version 6.0 [24] to obtain consensus and compared by alignment with NS3 sequences of representative reference strains of each HCV subtype obtained from the Los Alamos HCV Sequence Database [25]. To evaluate the presence of resistance mutations to DAAs, sequences from the HCV NS3 region were analyzed for substitutions in amino acid residues described in the literature associated or not with some degree of PI resistance: V36, Q41, F43, T54, Q80, S122, R155, A156, D168 and V170.

Statistical Analysis

Univariate analyses were used to associate hepatitis C subtypes and groups included in the study. Fisher’s exact test and Pearson chi-square were chosen when appropriate to test the significance level of associations, which was assessed at the 0.05 probability level. All analyses were performed using software EpInfo version 7.1 (Centers for Disease Control and Prevention, Atlanta, GA, USA).

Results

Overall RAS prevalence in this study was 13.7% (10/73). Among 73 patients enrolled in this study, 15 were treatment-experienced with first-wave PIs telaprevir/boceprevir (group 1) and 58 have not been treated with DAAs (group 2). The observation of amino acid residues of HCV NS3 in group 2 identified RASs at positions 36, 43, 54 and 80 in 6/58 (10.3%) patients. Regarding group 1 patients, RASs were identified in 4/15 (26.7%) at positions 36 (n = 3) and 155 (n = 1) in HCV 1a sequences.

Regarding HCV subtypes, the frequency of RAS in subtype 1b was 17.4% (4/23) while in subtype 1a was 12% (6/50). Primary mutations V36M and R155K were observed only in HCV subtype 1a, whereas T54S and Q80K were identified only in HCV subtype 1b. The positions 156 and 168, which are highly related to resistance to PIs, remained conserved in all 73 sequences. The association between amino acid mutations identified in NS3 region and resistance to protease inhibitors for HCV subtypes 1a and 1b is exposed in Table 3.

Regarding patients infected with HCV subtype 1a, 28.6% (4/14) from group 1 presented RASs while resistance strains were identified in 5.6% (2/36) of the individuals from group 2 (P = 0.044). For both groups, substitutions were observed at NS3 residue 36: 3 patients from group 1 presented RAS V36M and a combination of V36M+R155K was identified in another individual. For group 2, resistance strains V36L and V36M were found in two non-experienced patients.

In patients infected with HCV subtype 1b, RAS prevalence for group 2 was 18.1% (4/22). Identified mutations were F43V (1/22; 4.6%), T54S (1/22; 4.6%), Q80H (1/22; 4.6%) and Q80K (1/22; 4.6%). No mutations were found in the patient infected with HCV subtype 1b previously experienced with first-wave DAAs.

Discussion

In this study, 73 patients infected with HCV subtype 1a or 1b were evaluated to identify RASs previously reported in literature. Until the end of 2015, telaprevir and boceprevir were the unique PIs available for hepatitis C treatment in Brazil. However, the licensing of three drugs in 2015 (simeprevir, daclatasvir and sofosbuvir) allowed new combined therapeutic options. Simeprevir, a second-wave PI, was recommended for patients with non-advanced liver disease whereas the combination of DAAs daclatasvir (NS5A inhibitor) and sofosbuvir (NS5B nucleotide analogue inhibitor) was the main therapy for treatment-experienced patients especially those with decompensated cirrhosis. Recently, other PIs were included in clinical protocols for treating genotype 1-infected patients such as paritaprevir.
combined with dasabuvir/ombitasvir and grazoprevir with elbasvir.

As expected, RAS prevalence for treatment-experienced patients who failed therapy with first-wave, first-generation drugs telaprevir/boceprevir was higher compared to non-experienced patients likely due to drug-selective pressure. For non-responders to previous therapy, resistance substitutions represent a negative prognosis factor for new treatments with DAAs [26–28]. For 4 patients analyzed in the present study who failed previous therapy, the presence of RAS might have been the cause of non-response while for other telaprevir/boceprevir non-responder patients in whom no RASs were detected, other host or virological factors could have contributed to unsuccessful treatment. Medical records did not describe how long, after the treatment with first-wave drugs, serum samples from non-responder patients were collected. Thus, since it would be expected that wild-type strains could re-emerge as the major viral population some time after the absence of selective pressure imposed by drugs, a therapeutic failure due to the presence of RAS could not be excluded. However, previous DAAs non-response could also be associated with other factors, such as infection with HCV subtype 1a, cirrhosis and high infective viral load [29].

NS3 RAS prevalence in group 2 was lower in subtype 1a (5.6%) compared to subtype 1b (18.1%). The presence of resistance in patients of subtype 1b not exposed to drug-selective pressure suggests a prime infection with RASs strains. Considering both groups, this study identified higher proportion of RASs in HCV subtype 1b sequences when compared to subtype 1a (17.4% vs. 12%), a distinct pattern from that observed in a previous Brazilian study enrolling blood donors, where the presence of RAS in subtype 1a was significantly higher than in subtype 1b (20% vs. 8%) [30]. This might be related to differences in the study population (more than 20% of our samples were from individuals previously experimented with DAAs) and/or which amino acid positions were evaluated and taken in consideration when calculating RAS proportion between subtypes.

According to literature, RASs V36M, T54S, Q80K and R155K are considered primary resistance mutations to different PIs. RASs V36M and R155K can reduce susceptibility to recently approved PIs sitamaeprir, paritaprevir and grazopre-

v[r 18,31,32]. T54S is associated to resistance for first-wave telaprevir [33]. Q80K is highly associated with resistance to sitamaeprir [34].

The presence of RAS V36M in HCV strains could be related with therapeutic failure experimented by four patients in a previous treatment with telaprevir. The identification of this substitution in one patient from group 2 suggested a primary infection with a drug-resistant viral variant, an observation that warns for the circulation of resistant strains that could impact the effectiveness of DAAs in the near future. For all five patients with RAS V36M, combined therapy with new generation PIs should not be considered since V36M is associated with resistance to the majority of approved NS3 DAAs. Indeed, since it was already available in Brazil, treatment with sofosbuvir combined with daclatasvir was chosen and all five patients had similar treatment outcome which was undetectable HCV RNA after 12 weeks post-treatment. A study conducted a retrospective analysis to determine the prevalence of resistance mutations among telaprevir-
treated patients [33] and V36M mutation was identified in 28/232 (12%) patients which failed telaprevir therapy, thus demonstrating its importance as a mutation indicative of resistance whose poor prognosis does not reveal reliability in the use of first-generation PI. Barnard et al. [35] identified resistance mutations in non-responders to triple therapy with boceprevir/peg-IFN/RBV infected with subtype 1a and concluded that V36M can be a major cause of therapeutic failure with the use of first-wave PIs. Results from the present study demonstrated that mutations at position 36 were found in both DAA-experienced and non-experienced patients included in the study, which indicated that treatment with DAAs other than PI should be considered in order to minimize risk of resistance and achieve SVR in these patients.

Among RASs observed in patients from group 2, RAS T54S was identified in one patient infected with subtype 1b. This mutation had been shown to cause resistance to boceprevir and telaprevir, but not to simprevir [36]. This was confirmed here since this patient achieved SVR after 12 weeks of treatment with simprevir. The low prevalence of RAS T54S (4.6%) in patients not treated with DAAs was also reported in previous Brazilian studies [30,37].

In the present study, RAS Q80K was not observed in isolates of subtype 1a and was only detected in one subtype 1b sample from a group 2 patient with compensated hepatic cirrhosis, type 2 diabetes mellitus and systemic arterial hypertension. In 2016, this patient was asymptomatic and decided not to continue with other available DAA therapeutic options. Q80K is most frequently observed in subtype 1a isolates and is rarely detected in HCV subtype 1b [20]. Studies had reported the high prevalence of Q80K mutation in USA (37–47%) [38,39]. Sarrazin et al. [40] evaluated NS3 baseline RASs from 467 patients and results for Q80 polymorphisms demonstrated high prevalence for PI treatment-experienced patients (110/265; 41.5%) and PI treatment-naive patients (93/202; 46%). In contrast to data from other countries, Q80K prevalence in Brazil is low [30,37,41]. Therefore, due to low prevalence of this mutation in Brazilian strains reported in previous studies and corroborated here, there is no need to incorporate pretreatment resistance tests for infected patients with subtypes 1a and 1b of HCV in Brazil. Even with the identification of this variant, the use of other PIs is not limited since there is no evidence with resistance.

RAS R155K is related to resistance to first and second-wave PIs. A study reported a higher frequency of treatment failure for subtype 1a due to low genetic barrier to viral resistance when compared to subtype 1b [13]. Sarrazin et al. [36] described that combination of substitutions V36M + R155K induces high resistance to telaprevir and may inhibit drug action. In the present study, combination of mutations at loci 36 (V36M) and 155 (R155K) was identified in one telaprevir-experienced patient infected with HCV subtype 1a. After 12 weeks of therapy with telaprevir, viral load was 4.74 log10 IU/mL and it was decided to suspend the treatment. RAS R155K confers resistance to all available PIs for subtype 1a strains and new therapeutic options for this patient should target other non-structural HCV proteins. Indeed, in this case, a rescue therapy with NS5B and NS5A inhibitors was selected and HCV RNA was undetectable after 4 weeks.
Prevalence of baseline NS3 resistance-associated substitutions (RASs) on treatment with protease inhibitors in patients

Substitutions V36L, F43V and Q80H were identified in the present study. RAS V36L is associated with resistance to boceprevir [18]. In 2015, Brazilian clinical practice guidelines on the management of hepatitis C no longer included combined therapies with boceprevir or telaprevir as a treatment option. RAS V36L was identified in one subtype 1a patient from group 2. A 12-week therapy with simeprevir was initiated. V36L was not related with less susceptibility to this drug and SVR was achieved post-treatment.

Analysis of mutations associated with resistance among patients infected with HCV subtype 1b indicated the presence of RAS F43V in a DAA treatment-naive patient. To our knowledge, this is the first report of this mutation in vivo. Resistance profile for PIs was described in vitro and pointed out F43 locus as associated with resistance to simeprevir [18]. RAS Q80H, which can reduce susceptibility to simeprevir, was identified in one patient from group 2 infected with subtype 1b. Treatment with boceprevir was selected for this patient and Q80H did not influence treatment response since SVR was achieved after treatment. No Brazilian data have described this mutation among treatment-naive patients.

In conclusion, genetic data from HCV strains circulating in Brazil reported in this study pointed out that the use of simeprevir, paritaprevir, asunaprevir and grazoprevir has a high probability of being effective in our country. The genetic barrier for resistance to PIs can vary according to different genotypes and its specific polymorphisms; hence, this study will contribute to the knowledge of the impact of RASs for HCV subtypes 1a and 1b and your relation to first and second-generation PIs in strains circulating among Brazilian HCV chronic carriers.

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Authors’ contribution

V.D.C and F.C.A.M made the guarantor of the article.

V.D.C and F.C.A.M made the statistical analysis and wrote the paper; E.L. supervised the execution of the project and made critical reading of the manuscript; N.D. performed experiments; C.E.B.M and E.P.N. provided samples and data from patients; P.S.F.S. and L.L.L.X.S.R. provided clinical information. All co-authors approved the final version of the paper.

Disclosure of interest

The authors declare that they have no competing interest.

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References