CDKN1B V109G polymorphism a new prognostic factor in sporadic medullary thyroid carcinoma

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Abstract

Context: CDKN1B encodes the cyclin-dependent kinase inhibitor p27Kip1 and is mutated in multiple endocrine neoplasia-like syndromes. CDKN1B also harbors single nucleotide polymorphisms; the T/G transversion at nucleotide 326 (the V109G variant) has been reported to be protective in breast, hereditary prostate, and pancreatic tumors. Association of CDKN1B mutations or polymorphisms with sporadic medullary thyroid carcinoma (MTC) has not been investigated yet.

Objective and design: We screened germline DNA from 84 patients affected by sporadic MTC and 90 healthy age- and gender-matched controls for CDKN1B mutations or polymorphisms by PCR amplification and sequencing of the amplicons. We also tested all germline and 50 tumor tissue DNA for RET proto-oncogene mutations. Computed tomography, ultrasound scans, and serum calcitonin were carried out before surgery and during the follow-up and associated with CDKN1B polymorphism and disease remission.

Results: The T/G transversion at nucleotide 326 was the only DNA variation detected. The overall frequency of the T/G and G/G alleles in combination was 46.4%. This variant (V109G) was correlated with post-operative calcitonin levels in the normal range and biochemical remission. Conversely, the wild-type (T/T) allele was associated with post-operative calcitonin levels above normal and a higher risk to develop clinical recurrence and distant metastases. Somatic RET mutations were significantly associated with a more aggressive behavior especially in wild-type allele-bearing patients.

Conclusions: Collectively, in sporadic MTC, the CDKN1B V109G polymorphism correlates with a more favorable disease progression than the wild-type allele and might be considered a new promising prognostic marker.

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Introduction

CDKN1B encodes the cyclin-dependent kinase (Cdk) inhibitor p27Kip1, a regulatory protein that controls the progression from the G1 to the S phase of the cell cycle by interacting with cycline/Cdk2 and cyclinD1/Cdk4 complexes (1, 2). Loss-of-function mutations have been described and contribute to tumorigenesis. Specifically, mutations of the p27Kip1 (Cdkn1b) gene in rat are associated with the development of a MEN X syndrome characterized by a clinical picture that overlaps MEN 1 and MEN 2 syndromes ((3) and references therein). In humans, a MEN 1-like syndrome has recently been described and referred as MEN 4 (3, 4). No mutations in CDKN1B have been found in 16 patients with a MEN 1 phenotype without MEN 1 mutations (5, 6), suggesting that CDKN1B germline mutations can predispose to the development of endocrine tumors in humans (3, 4, 7). A total of 21 single nucleotide polymorphisms in CDKN1B have also been described, 11 of which have low allelic frequency (5%) and 9 occur within the non-coding regions of the gene (8, 9). A single nucleotide polymorphism (T/G) at position 326 that causes a glycine for valine amino acid substitution at codon 109 of the mature protein has variably been associated with cancer risk and progression in several tumors including...
prostate (10, 11), breast (12, 13), oral squamous cell (14), and pancreatic carcinomas (15, 16). The frequency of the variant in such tumors ranges from 6 to 40% and appears not to be significantly different from controls (10–16). So far, no evidence has been provided to support the association of CDKN1B mutations or polymorphisms with tumor progression and outcome in medullary thyroid carcinoma (MTC). This tumor arises from the neural crest-derived calcitonin-producing or parafollicular C cells of the thyroid gland (17, 18) and occurs sporadically in about 75% of cases or as a component of the autosomal dominant-inherited MEN 2 syndromes or as a unique manifestation of the familial MTC (FMTC) (19–22). Mutations of the RET proto-oncogene are responsible for the large majority of MEN 2 and FMTC cases (23, 24) and for a proportion of sporadic MTCs. It is still unknown whether another frequently mutated gene or multiple low frequency mutated genes are responsible for MTCs not bearing RET mutations. Moreover, the clinical phenotype of sporadic and inherited MTCs is heterogeneous even in the presence of the same mutation: also the relationship with the clinical course and prognosis may be variable and the molecular mechanisms are still under investigation. The search of new biomarkers that can easily and objectively be determined can help in MTC diagnosis and management. Since the V109G polymorphism has been associated with endocrine tumors, in this study, we sought to search for CDKN1B mutations or polymorphisms, specifically for the V109G polymorphism, in a large sample of sporadic MTCs negative for germline RET mutations and correlate its presence with disease progression.

Subjects and methods

Study subjects

Eighty-four consecutive patients, 34 males and 50 females, diagnosed with sporadic MTC at the participating institutions from 1999 to 2009, were enrolled in this study. They were selected on the basis of a lack of known RET germline mutations, family history of thyroid diseases, negative clinical, laboratory, and family data for any other malignancy or predisposition to develop it. No loss of follow-up or study end-data was observed in our subjects. Cancer-related death occurred in a single patient. Patients’ mean age at diagnosis was 46.1 ± 13.2 years, and the median follow-up was 84.0 months. All affected individuals were subjected to total thyroidectomy and central neck dissection following standard procedures. Lymphadenectomy of the lateral compartment(s) was performed during the first surgery if node metastases were already diagnosed or detected during the intervention. Ninety subjects, age (± 5 years) and gender-matched with the enrolled patients, served as control. Thirty-six male and fifty-four female individuals aged <60 year old, with no history of MTC, basal serum calcitonin levels <10 pg/ml, and absence of thyroid nodules, were recruited among the medical and paramedical personnel of the institutions participating in the present study. These criteria were designed to ensure that all of them had a minimal risk of having or ever developing an MTC. After informed consent was obtained, each subject was interviewed using a pretested questionnaire to obtain information on medical history, lifestyles, and family history of cancer up to first-degree relatives. Based on these information, the control group does not include individuals with history of breast, hereditary prostate and pancreatic tumors, or any other cancer. All patients and controls were of European descent with a nationwide distribution. Informed consent for all genetic screenings, blood samples handling and processing, and other clinical procedures was provided by all investigated subjects in accordance with the guidelines approved by the local ethical committee. Serum calcitonin and carcinoembryonic antigen (CEA) were employed as MTC tumor markers; serum calcitonin was measured before and routinely after surgery, recording the trend of the last few determinations, for the long-term surveillance, according to the recommendation of the American Thyroid Association Guidelines Task Force (25).

Serum calcitonin assay

Serum calcitonin was measured by a commercially available IRMA test (Byk Gulden Italia S.p.A., Milan, Italy) according to the manufacturer’s instructions, as previously described (26).

Analysis of the CDKN1B V109G polymorphism and RET mutation

The analysis was performed on germline DNA from patients and control blood samples. CDKN1B exons 1 and 2 were PCR amplified using the following oligonucleotides as primers: exon1 fw: GTAGGGGCTTTTGTTTT, rev: GCCAGGTAGCCTGAACACC; exon2 fw: GTAGGGGGCTTTTGTTTT, rev: ACAGGGAAACGACC- TTCTCTAC; and the following amplification conditions: 5 m for the initial denaturation step at 95 °C followed by 35 cycles of 15 s at 94 °C, 30 s at 60 °C, 1 m at 72 °C, and a final 5 m step at 72 °C for the final extension. RET proto-oncogene mutations were searched by PCR amplification of exons 8, 10, 11, 13, 14, 15, and 16 on germline DNA from all patients and somatic DNA from 50 tumor samples using the primers reported in the literature (27, 28). All PCR products were isolated and subjected to automatic sequence analysis (Applied Biosystem Bioprism 3100, Foster City, CA, USA). The tests were performed on at least three different germline DNA preparations obtained from independent drawings. Also the tumor tissue analysis was carried out on two different DNA preparations.
Statistical analysis

All statistical analyses were carried out with the SPSS (version 15.0) for Windows (SPSS, Inc., Chicago, IL, USA). The χ², Fisher exact, or the Spearman test was employed to assess the association of the V109G polymorphism with several biochemical, clinico-pathological parameters and RET mutations. The influence of the p27Kip1 polymorphism and other clinico-pathological variables on disease-free survival (DFS) was evaluated by the Kaplan–Meier method; differences were analyzed with the log-rank test. Cox regression models were used to evaluate the effect of the CDKN1B polymorphism on patients’ outcome after adjusting for other covariates or potential confounders. Several clinico-pathological variables were included in the final multiple regression model whereby hazard rate (HR), 95% confidence interval (95% CI), and significance levels were estimated. A stepwise selection procedure was used to identify those markers that independently predict disease progression. The analysis of variance between groups was performed by ANOVA. Data were reported as mean ± s.e.m., and the mean values were compared using the Student’s t-test or the Mann–Whitney U test. Results were considered statistically significant when a P ≤ 0.05 was obtained.

Results

CDKN1B polymorphism detection in MTC patients and control subjects

To determine whether CDKN1B mutations or single nucleotide polymorphisms are associated with MTC disease progression, we genotyped n = 84 patients affected by sporadic MTC and n = 90 matched controls. The analysis was carried out on germline DNA by PCR-amplifying exons 1 and 2 of the gene, followed by automatic sequencing. The only gene variation was the T/G transversion at nucleotide 326. The arrow in the lower electropherogram denotes two overlapping peaks, indicative of the presence of two different nucleotides at that position of the sequence. The change causes a valine (GTC) for glycine (GGC) substitution at position 109 of the mature protein. (Fig. 1A). Only a slight, borderline difference in the frequency of the wild-type (T/T, 53.6%) and polymorphic alleles (T/G 45.2%, G/G 1.2%) was found between patients and controls (P = 0.048; Fig. 1B). The search for RET mutations was performed by amplifying exons 8–16 from germline DNA of all patients and from somatic DNA extracted from 50 available tumor tissues and sequencing the obtained amplicons. All patients were negative for RET germline, whereas RET somatic mutations were found in 13/50 tumor tissue DNA (26%), a percentage slightly lower than that reported in the literature (25, 26). They were equally distributed between wild-type- and polymorphism-bearing patients, 25.9 vs 26.1% respectively. Twelve cases (24%) had the M918T mutation, and a single patient had the C634W mutation (2%). The RET genetic test data do not rule out mutations that may be present in the exons not investigated both in the germline and in the somatic DNA.

Clinico-pathological parameters, CDKN1B polymorphism, and serum calcitonin levels

Patients were subdivided into two groups, WT (T/T genotype) and POL (a combination of T/G and G/G genotypes) according to the presence of the CDKN1B wild-type or polymorphic allele respectively. The CDKN1B genetic status and some of the clinico-pathological characteristics of the patients analyzed are reported in Table 1. Patients’ age, gender, tumor stage, and last post-operative serum calcitonin levels from WT were compared with POL patients (Table 1). The age at diagnosis was lower in POL than in WT patients (P = 0.026). An association was found between the CDKN1B genetic status and the T stage, i.e. wild-type allele-bearing patients had a more advanced (T3/T4) tumor than patients with the polymorphic allele.

Figure 1 Identification of the CDKN1B V109G single nucleotide polymorphism and its allelic frequency in MTC patients and controls. (A) Direct sequencing of CDKN1B-amplified exon 1 from two patients’ DNA shows either the wild-type sequence or the T/G transversion at nucleotide 326. The arrow in the lower electropherogram denotes two overlapping peaks, indicative of the presence of two different nucleotides at that position of the sequence. The change causes a valine (GTC) for glycine (GGC) substitution at position 109 of the mature protein. (B) Relative frequency of the T/G (V109G) polymorphism in patients and control individuals. Full colour version of this figure available via http://dx.doi.org/10.1530/EJE-10-0929.
considered (234 vs 7.7 pg/ml, Fig. 2). Collectively, these
in POL patients when the median absolute values were
post-operative levels were significantly higher in WT than
CDKN1B
presence of the
between last post-operative serum levels and the
The calcitonin data suggest a robust inverse relationship
CDKN1B
patients bearing the
in MTC in
POL cases (49 vs 18%;
(71x100)
(71x100)
P
59%;
P
in 13 WT subjects compared to 23 POL patients (29 vs
operative serum calcitonin was within the normal range
recurrence and calcitonin levels above normal range
polymorphism-bearing patients with a longer DFS ( Fig. 3 ).
second group included patients with biochemical/clini-
PT level values ranging from 10 to max 150 pg/ml were considered in biochemical recurrence and were equally distributed between the POL and WT groups (Table 1). For statistical purpose, we stratified the patients into two groups: the first group included patients with biochemical remission (with calcitonin levels in the normal range ≤10 pg/ml); the second group included patients with biochemical/clini-
cal recurrence and calcitonin levels above normal range
(> 10 pg/ml). Kaplan–Meier analysis performed on the
two groups showed a significant association of poly-
mosis-bearing patients with a longer DFS (Fig. 3).
Somatic RET mutations (13/50 cases analyzed) were equally distributed between the WT and POL groups

### Table 1 Relationship between CDKN1B polymorphism, clinical and biochemical data of the MTC patients investigated. Patients enrolled in this study were divided into two groups. The WT group carries the CDKN1B T/T allele corresponding to the valine at position 109 of the mature protein. The POL group carries both the T/G and G/G alleles corresponding to the amino acid glycine of the mature protein (V109G). CT last, last post-operative calcitonin levels.

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*Significant at 0.05 level; †significant at 0.01 level.
*Recommendations 73 and 74 given in Kloos et al. (25).

(P = 0.05). Interestingly, a strong correlation was found between the presence of distant metastases and the CDKN1B wild-type allele (78 vs 22%, Table 1, P = 0.0001). The pre-operative serum calcitonin did not show significant differences between the two groups (Fig. 2; P = 0.988). In contrast, the last post-operative serum calcitonin was within the normal range in 13 WT subjects compared to 23 POL patients (29 vs 59%; P = 0.0001), whereas it was frankly elevated (>150 pg/ml) in 22 WT cases compared with only 7 POL cases (49 vs 18%; P = 0.0001) (Table 1 and Fig. 2). The calcitonin data suggest a robust inverse relationship between last post-operative serum levels and the presence of the CDKN1B polymorphism. Moreover, only post-operative levels were significantly higher in WT than in POL patients when the median absolute values were considered (234 vs 7.7 pg/ml, Fig. 2). Collectively, these data suggest a more aggressive course of the MTC in patients bearing the CDKN1B wild-type allele as compared with the polymorphism-bearing patients.

Relationship between RET mutations and recurrence rate in MTC patients

The overall clinical follow-up was available for all patients and determined starting from the day of the first surgery with a median of 84.0 months. All 22 patients bearing the CDKN1B wild-type allele had serum calcitonin levels > 150 pg/ml and experienced clinical recurrences with symptomatic progressive loco-regional disease. Thirteen wild-type allele-bearing patients, with serum calcitonin levels in the normal range, were in biochemical remission with no signs of persistent or recurrent disease. On the other hand, 23 patients bearing the polymorphism had serum calcitonin levels in the normal range and were in biochemical remission as evidenced by lack of signs or symptoms, imaging data, and long-term follow-up. In contrast, only seven polymorphism-bearing patients had serum calcitonin levels above 150 pg/ml and presented clinical recurrences. Patients with calcitonin values ranging from 10 to max 150 pg/ml were considered in biochemical recurrence and were equally distributed between the POL and WT groups (Table 1). For statistical purpose, we stratified the patients into two groups: the first group included patients with biochemical remission (with calcitonin levels in the normal range ≤10 pg/ml); the second group included patients with biochemical/clini-
cal recurrence and calcitonin levels above normal range
(> 10 pg/ml). Kaplan–Meier analysis performed on the
two groups showed a significant association of poly-
mosis-bearing patients with a longer DFS (Fig. 3).
Somatic RET mutations (13/50 cases analyzed) were equally distributed between the WT and POL groups

Figure 2 Serum calcitonin levels in patients carrying the CDKN1B wild-type or polymorphic allele. The pre- and last post-operative calcitonin levels are represented by box plot for each group of patients, carrying either the CDKN1B wild-type (T/T genotype) or the polymorphic allele (a combination of T/G and G/G genotypes) respectively. The edges of the boxes are the interquartile range box, and lines in the box represent the median value. CT levels, calcitonin levels; CT pre WT, pre-operative calcitonin levels in wild-type group; CT pre POL, pre-operative calcitonin levels in polymorphic group; CT last WT, last post-operative calcitonin levels in both groups.

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and were associated with a more aggressive course of the disease especially in CDKN1B WT allele-bearing patients, as documented by the presence of distant metastases, advanced T stage (T3/T4), and higher calcitonin levels (Table 2). No relationship was found for other clinico-pathological variables such as gender, lymph node involvement, and patients' age at diagnosis (data not shown).

**Univariate and multivariate analysis**

To assess whether the CDKN1B V109G polymorphism and the clinico-pathological variables have a prognostic significance, Cox regression analysis was performed for 83 patients, because only one cancer-related death occurred in our series (Table 3). Univariate analysis showed that the CDKN1B polymorphism significantly correlates (HR calculation with a 95% CI) with DFS when patients were stratified as in biochemical remission or in biochemical/clinical recurrence with calcitonin levels above normal range. This indicates that patients carrying the CDKN1B wild-type allele have a relative higher risk of recurrence than patients carrying the polymorphism. Multivariate model showed that CDKN1B genetic status preserves a prognostic significance when adjusted for tumor stage ($P < 0.05$; Table 3). No significant effects were observed when other variables such as age at diagnosis or gender were included in the multivariate model. In a similar analysis, performed taking into account only the subgroup of $n=50$ patients analyzed for somatic RET mutations, the polymorphism significantly correlated with patients' outcome when adjusted for RET mutations ($P$ interaction $< 0.05$, Table 3). These data suggest that the CDKN1B polymorphism could be a potential prognostic factor for disease progression in MTC patients.

**Discussion**

Over the past years, great attention has been paid to the new achievements in understanding several aspects of MEN 2 syndromes, including the identification of new clinical entities and genes. A MEN 1-like syndrome, defined MEN X, has been described in rats with a clinical picture that partially overlaps MEN 1 and MEN 2A phenotypes (3). Pellegata et al. identified a nonsense mutation in the CDKN1B human gene as a causative alteration that results in a dramatic reduction of the protein. These patients are considered affected by MEN 4 and present clinical features resembling MEN 1.

![Relationship of the CDKN1B genetic profile with MTC remission. Kaplan–Meier analysis was referred to the CDKN1B genetic status. The $P$ value reported in the graph was obtained with the log-rank test. Wild-type group refers to T/T genotype; polymorphic group comprises a combination of T/G and G/G alleles. WT, CDKN1B wild type; POL, CDKN1B V109G polymorphism.](image)

Table 2 Relationship between CDKN1B polymorphism, somatic RET mutations, and some clinico-pathological features of the MTC patients. Somatic RET mutations were analyzed in a subgroup of $n=50$ patients and were found in 13/50 patients (26%). Twelve cases (24%) had the M918T, and a single patient (2%) had the C634W mutation. The CDKN1B POL group comprises the combination of T/G and G/G alleles; the WT group refers to the T/T allele.

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</table>

Last CT, last post-operative calcitonin levels; RET+, RET mutation positive; RET−, RET mutation negative. *Significant at 0.05 level.
symptom (3, 4). More recently, CDKN1B germline mutations have been described in multiple endocrine tumors (7). CDKN1B also harbors numerous polymorphisms that in several tumors have been variably associated with disease progression and patients’ outcome (11–16). Specifically, the V109G polymorphism has been shown to have a protective effect on the overall patients’ survival in sporadic pancreatic cancer (16). Subsequent surveys on tumors of different origins have produced conflicting results, leaving unanswered the question as to whether the CDKN1B V109G polymorphism is associated with a better or worse prognosis (11–15). In this study, we investigated whether CDKN1B mutations or polymorphisms could be associated with MTC disease progression. The only DNA variation detected was a T/G transversion at position 326 that corresponds to the valine to glycine substitution at codon 109 of the mature protein. A borderline difference in the frequency of the wild-type and polymorphic alleles was found between patients and controls. Patients were selected to exclude those presenting C-cell hyperplasia, those related to inherited MEN 2 syndromes or FMTCs, or those associated with paragangliomas in various localizations or any other tumor. The patients were enrolled during the last 10 years (1999–2009) on the basis of the initial diagnosis and surgery performed at least 2–4 years earlier to have a sufficient long time span to evaluate the incidence of recurrence or the extent of the survival. We provide evidence that MTC patients bearing the CDKN1B V109G polymorphic allele have more frequently last post-operative basal serum calcitonin levels in the normal range than those bearing the wild-type allele (Fig. 2). Interestingly, the POL patients have a significantly lower rate of clinical recurrence, distant metastases, and hence a better outcome, suggesting that the V109G polymorphism may modulate MTC progression and be protective against recurrences (Table 1). Consistent with this, patients bearing the polymorphism, although presenting moderately elevated calcitonin levels, have a more indolent disease course than those with the wild-type allele and similar calcitonin levels. Somatic RET mutations appear to worsen disease progression in wild-type allele-bearing patients, as documented by a shorter DFS, stage of the tumor, and presence of distant metastases. In patients bearing the polymorphic allele, instead, RET mutations do not have the same impact confirming that the polymorphic allele exerts a protective effect against a more aggressive disease. In line with this, a significant interaction of the CDKN1B V109G polymorphism with somatic RET mutations was observed in a multivariate analysis. Laboratory and imaging data as well as clinical evidences supported this hypothesis. This is the first study reporting that the CDKN1B V109G polymorphism with somatic RET mutations was observed in a multivariate analysis. Laboratory and imaging data as well as clinical evidences supported this hypothesis. This is the first study reporting that the CDKN1B V109G polymorphism with somatic RET mutations was observed in a multivariate analysis. Laboratory and imaging data as well as clinical evidences supported this hypothesis.

### Table 3

Univariate and multivariate Cox’s proportional hazard analysis in MTC patients. Cox regression analysis was performed on all available patients (n = 83) or on the subgroup of patients analyzed for RET somatic mutations (n = 50). The CDKN1B WT group refers to the T/T allele; the POL group comprises the combination of T/G and G/G alleles.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>All patients (n = 83)</th>
<th></th>
<th>Subgroup (n = 50)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate</td>
<td>Multivariate adj. HR</td>
<td>Univariate</td>
<td>Multivariate adj. HR</td>
</tr>
<tr>
<td>CDKN1B (WT versus POL)</td>
<td>1.81 (0.98–3.31) 0.048*</td>
<td>1.96 (1.03–3.73) 0.04*</td>
<td>2.02 (0.89–4.59) 0.067</td>
<td>2.42 (1.04–5.60) 0.038*</td>
</tr>
<tr>
<td>Gender (M versus F)</td>
<td>1.43 (1.03–3.25) 0.070</td>
<td></td>
<td>1.89 (1.14–5.71) 0.160</td>
<td></td>
</tr>
<tr>
<td>Age (≤ 45 vs &gt; 45)</td>
<td>1.11 (0.62–1.98) 0.730</td>
<td>1.28 (0.59–2.75) 0.528</td>
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<tr>
<td>Tumour stage (II/III vs I/II)</td>
<td>1.72 (1.23–2.40) 0.002 †</td>
<td>1.67 (1.18–2.35) 0.004 †</td>
<td>2.35 (1.46–3.77) 0.000 †</td>
<td></td>
</tr>
<tr>
<td>RET mutation (+ve versus –ve)</td>
<td>2.23 (1.03–4.84) 0.042*</td>
<td>2.68 (1.21–5.92) 0.015*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

+ve, positive; −ve, negative; adj. HR, adjusted HR; WT = CDKN1B wild-type-bearing patients; POL = CDKN1B polymorphism-bearing patients. *Significant at 0.05 level; †Significant at 0.01 level.
confirm this hypothesis. The CDKN1B polymorphism may also be in linkage disequilibrium with other functional polymorphisms that affect either the expression or the activity of enzymes involved in tumorigenesis.

In conclusion, the CDKN1B V109G polymorphism might influence the clinical course of patients presenting sporadic MTC. The assessment of the CDKN1B genotype might be a new tool for MTC prognosis along with other known markers, such as the last postoperative serum calcitonin and RET mutations’ detection. Further studies will allow a better understanding of the molecular basis of the proposed protective effect of the CDKN1B polymorphism in sporadic MTC.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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