




Autophagy ATG16L1 rs2241880 impacts the colorectal cancer risk: A case-control study

Leila Jamali¹ | Hossein Sadeghi²  | Mohammad-Reza Ghasemi¹  |
Roohollah Mohseni³ | Ehsan Nazemalhosseini-Mojarad⁴ | Vahid Reza Yassae² |
Pegah Larki² | Mohammad Reza Zali⁴ | Reza Mirfakhraie^{1,5} 

¹Department of Medical Genetics, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

²Genomic Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

³Clinical Biochemistry Research Center, Basic Health Sciences Institute, Shahrekord University of Medical Sciences, Shahrekord, Iran

⁴Department of Gastrointestinal Cancer, Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran

⁵Hematopoietic Stem Cell Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Correspondence

Reza Mirfakhraie, Department of Medical Genetics, School of Medicine, Shahid Beheshti University of Medical Sciences, Koodakyar St., Velenjak Ave, Chamran Highway, Tehran 19395-4719, Iran.
Email: reza_mirfakhraie@yahoo.com

Hossein Sadeghi, Genomic Research Center, Shahid Beheshti University of Medical Sciences, Yeman St, Chamran Highway, Tehran, Iran.
Email: Hsadeqi86@gmail.com

Funding information

The authors would like to thank the "Genomic Research Center, Shahid Beheshti University of Medical Sciences" for financial support

Abstract

Background: Despite many efforts to discover the important role of the autophagy process in the pathogenesis of colorectal cancer (CRC), the exact involved molecular mechanism still remains to be elucidated. Recently, a limited number of studies have been employed to discover the impact of autophagy genes' variants on the development and progression of CRC. Here, we evaluated the association between two single-nucleotide polymorphisms (SNPs) in the main components of the autophagy genes, ATG16L1 rs2241880, and ATG5 rs1475270, and the CRC risk in an Iranian population.

Methods: During this investigation, a total of 369 subjects, including 179 CRC patients and 190 non-cancer controls have been genotyped using Tetra-primer amplification refractory mutation system-polymerase chain reaction (TP-ARMS-PCR) method.

Result: The results demonstrated that the T allele of the ATG16L1 rs2241880 was significantly associated with the increased risk of CRC in the studied population (OR 1.64, 95% CI: 1.21–2.22, $p = 0.0015$). Moreover, ATG16L1 rs2241880 TT genotype increased the susceptibility to CRC (OR 3.31, 95% CI: 1.64–6.69, $p = 0.0008$). Furthermore, a significant association was observed under the recessive and dominant inheritance models ($p = 0.0015$ and $p = 0.017$, respectively). No statistically significant differences were found in the ATG5 rs1475270 alleles and genotypes between the cases and controls.

Conclusion: The results of the present study may be helpful concerning the risk stratification in CRC patients based on the genotyping approach of autophagy pathways and emphasize the need for further investigations among different populations and ethnicities to refine our conclusions.

KEYWORDS

ATG16L1, ATG5, autophagy, colorectal cancer (CRC), single-nucleotide polymorphism (SNP)

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2021 The Authors. *Journal of Clinical Laboratory Analysis* published by Wiley Periodicals LLC.

1 | INTRODUCTION

Colorectal cancer (CRC), with a high rate of mortality and morbidity, imposes a large social and economic burden in the world.^{1,2} It has been estimated that approximately 1,400,000 new cases suffer from CRC worldwide.³ Although, the figures for the incidence and mortality rate could be varied depending on the race and ethnicity, as well as geographical features, generally, CRC is the third most frequent cancer all around the world.^{1,3} Moreover, in Iran, CRC is the third most prevalent neoplasia.⁴ Considering the etiology of CRC, multiple factors contribute to the disease development consisting of both genetic and non-genetic elements, including nutrition patterns, alcohol consumption, body mass index, physical inactivity and smoking. However, to discover all exact factors involved in the pathogenesis of CRC, different aspects of the CRC must be considered.⁵⁻⁹ Clearly, in CRC, more than one pathway is related with the tumor to undergo tumorigenesis.¹⁰ Autophagy is a highly evolutionary conserved catabolic process implicating in human health and diseases and dysregulated in a wide spectrum of human cancers, including CRC.¹¹ Although autophagy is considered as a tumor-suppressive mechanism against the early stage of tumorigenesis due to providing the cellular homeostasis; however, in some context, it could promote tumor survival.^{12,13} The elucidation of the functional relevance of the autophagy pathway and different aspects of CRC, including development, progression, and metastasis still remain a challenging issue.¹⁴⁻¹⁶ This multistep catabolic process is mediated by ubiquitin-like systems consist of multiple autophagy-related proteins (ATG).¹⁷ Single-nucleotide polymorphisms (SNPs) play a role in the disease mechanisms and effect the development of the precision medicine.¹⁸ Given the importance of the autophagy pathway in the etiology of neoplasia, recently, some emerging data have supported that the SNPs in main lysosomal pathway genes associate with the risk of multiple cancers, including breast cancer, melanoma, gastric cancer, prostate cancer, and colorectal cancer.¹⁹⁻²² Hence, we selected and performed genotyping of two SNPs in autophagy core genes, *ATG16L1* rs2241880 and *ATG5* rs1475270, to evaluate the potential role of these polymorphisms on the risk of CRC in an Iranian population.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

This survey was performed according to the Declaration of Helsinki and approval of the Ethics Committee of Shahid Beheshti University of Medical Sciences (SBMU). Written informed consent was obtained from all participants.

2.2 | Subjects

During this case-control investigation, 369 participants, including 179 patients, and 190 non-cancer controls were recruited. All participants were referred from Taleghani General Hospital, Tehran, Iran, between 2006 and 2015. First, based on a structured interview, the clinicopathologic information was gathered according to the medical records and interview, including age, sex, family history of cancer, ethnicity, literacy, occupation, marital status, stage, and grade. Furthermore, the confirmation of all affected diagnosed with CRC was checked based on their medical records involving clinical examination, colonoscopy, and histopathological features. Some exclusion criteria were considered, including a history of hereditary or other malignant diseases.

2.3 | DNA extraction and genotyping

Genomic DNA was extracted from lymphocytes of peripheral blood from each subject using the standard salting-out method.²³ Genotyping was performed by using Tetra-primer amplification refractory mutation system-polymerase chain reaction method (TP-ARMS-PCR). Primer1 online software²⁴ was utilized to design primers for genotyping of both *ATG16L1* rs2241880 and *ATG5* rs1475270 polymorphisms. PCR reactions were carried out on a GeneTouch thermocycler (BIOER) instrument using a total volume of 20 μ l, including 10 μ l Taq DNA Polymerase 2X Master Mix Red (Amplicon), 1 μ l of each primer (10 pmol), 1 μ l genomic DNA (100–200 ng), and 5 μ l PCR grade water. The cycling conditions consisted of: 5 min for initial denaturation at

TABLE 1 Primer sequences and TP-ARMS-PCR conditions for *ATG16L1* rs2241880 and *ATG5* rs1475270 amplification

SNP	Primer	Primer sequence	Amplicon size (bp)	TA
rs2241880	FI	CCTCACTTCTTTACCAGAACCAGGATGCGC	181 (C allele)	55°C
	RI	CCCAGTCCCCCAGGACAATGTGGCTA	107 (T allele)	
	FO	GGGTTCTGGGGCTGAAGCATACTTACGAA	235	
	RO	GGCAGTCTGCTTAAGCAAGGTCGCTTGG		
rs1475270	FI	TGATTTTAAAAGTCATGGAAAATTA CTG	209 (G allele)	51.5°C
	RI	TTTCATACTTTTCATGGTTATTCTCATTT	275 (A allele)	
	FO	AATCTACAAAACACCTGGAAATAAAAAG	426	
	RO	CCCTGGTTTATAGTAAAGTAGTTTGAC		

Note: The nucleotide specificity is indicated in parentheses.

Abbreviations: bp, base pair; F, forward; I, inner; O, outer; R, reverse; SNP, Single-nucleotide polymorphism; TA, annealing temperature.

95°C, followed by 32 cycles of denaturation at 95°C for 30 s, annealing temperature as presented in Table 1 for 1 min, and extension at 72°C for 1 min. The final elongation step was conducted at 72°C for 5 min. Table 1 describes the primer sequences and TP-ARMS-PCR conditions for amplification of ATG16L1 rs2241880 and ATG5 rs1475270 alleles. After PCR amplification, 2% of agarose gel electrophoresis containing RedSafe stain (iNtRON) in 0.5X tris/borate/EDTA (TBE) was used to separate the amplification products.

2.4 | Statistical analysis

Statistical analyses for allele count and genotype frequencies were performed based on Hardy-Weinberg equilibrium approach using online software, including SNPstats²⁵ and MEDCALC online software available from (https://www.medcalc.org/calc/odds_ratio.php). Statistically significant was considered according to *p*-value less than 0.05.

3 | RESULTS

Totally, 179 patients diagnosed with CRC (90 males and 89 females) with an average age of 52.1 ± 9.8 years were recruited in the current study. The sex- and age-matched control group was composed

TABLE 2 Demographic data and clinical characteristics of the study participants

Characteristics	CRC (n = 179) patients	Non-cancer controls (n = 190)
Median age, years	52.1 ± 9.8	51.2 ± 9.1
Gender		
Male	90	98
Female	89	92
Primary tumor location		
Colon	60%	
Rectum	27%	
Cecum	13%	
Differentiation		
Well-differentiated	35%	
Moderately differentiated	27%	
Poorly differentiated	7%	
Not determined	31%	
Cigarette smoking		
No	85%	
Yes	15%	
Clinical stages, TNM		
I	17%	
II	47%	
III	29%	
IV	7%	

of 190 non-cancerous subjects (98 males and 92 females) with the mean age of 51.2 ± 9.1 years. All controls were the same ethnic background. Table 2 describes the demographic and clinical information of the studied individuals.

Table 3 demonstrates genotypes and allele frequencies for ATG16L1 rs2241880 and ATG5 rs1475270 polymorphisms in CRC and control subjects. Both SNPs, which were genotyped in the control group, were consistent with those expected from the Hardy-Weinberg equilibrium model. We assessed the association between the risk of CRC and polymorphic variants at ATG16L1 rs2241880 and ATG5 rs1475270 in recessive and dominant inheritance models. The data obtained from statistical analysis illustrated that the T allele of ATG16L1 rs2241880 increased the risk for CRC in the studied population (OR 1.64, 95% CI: 1.21–2.22, *p* = 0.0015) and ATG16L1 rs2241880 T/T genotype was associated with the increased risk of CRC in the patients (OR 3.31, 95% CI: 1.64–6.69, *p* = 0.0008). Moreover, the T/T+ C/T vs. C/C genotypes were statistically associated with an enhanced CRC risk (OR = 1.71; 95% CI: 1.10–2.65, *p* = 0.017) as well as T/T vs. C/C+ C/T (OR = 2.76; 95% CI: 1.44–5.30, *p* = 0.0015). No significant association was observed between ATG5 rs1475270 alleles and genotypes with the CRC risk in any of the inheritance models (Table 3).

4 | DISCUSSION

Despite a considerable improvement in the recent biological and molecular knowledge of autophagy in the field of cancers, the exact molecular mechanisms involving in CRC based on cell-type and context-dependent remain poorly understood.^{12,14} To date, a limited number of studies have paid attention to the association of genetic variations in the autophagy genes and their impact on the cancer susceptibility and clinical outcome of malignancies in the diverse populations.^{19,20,26} In the present study, we evaluated the association of autophagy-related genes, ATG16L1 rs2241880 and ATG5 rs1475270, with CRC risk in an Iranian population. Our results revealed that the ATG16L1 rs2241880 T allele served as a CRC-risk allele in the studied population (*p* = 0.0015) and ATG16L1 rs2241880 TT genotype was associated with elevated susceptibility of CRC (*p* = 0.0008). Previously, Grimm WA and colleagues showed that the ATG16L1 Ala/Ala genotype was associated with longer overall survival and reduced metastasis in human colorectal cancer.²⁷ In addition, Nicoli ER et al.²⁸ reported an association between ATG16L1 genotypes and tumor stages in CRC patients. They found that the patients carrying GG genotype were at higher risk for developing CRC. The relation between rs2241880 and unfavorable clinical outcomes for colorectal cancer was also reported in Chinese population.²⁶ Fernández-Mateos et al.²⁹ reported the association between ATG16L1 rs2241880 CC genotype and increased susceptibility of oral carcinoma in an Spanish population. While, Diler et al. observed no association between this variant and susceptibility to prostate and bladder cancers in the Turkish population.²¹ According to HaploReg³⁰ and PROMO³¹ software, it is suggested

Genotype/Allele	Case N (%)	Control N (%)	OR (95% CI)	p-Value
ATG16L1 rs2241880 T>C				
C/C	52 (30.1)	76 (42)	1 (reference)	
C/T	87 (50.3)	90 (49.7)	1.41 (0.89–2.24)	0.14
T/T	34 (19.6)	15 (8.3)	3.31 (1.64–6.69)	0.0008
T/T+ C/T vs. C/C			1.71 (1.10–2.65)	0.017
T/T vs. C/C+C/T			2.76 (1.44–5.30)	0.0015
C	191 (55)	242 (67)	0.61 (0.45–0.83)	0.0015
T	155 (45)	120 (33)	1.64 (1.21–2.22)	0.0015
G/G	88 (50.6)	78 (45.4)	1 (reference)	
ATG5 rs1475270 A>G				
A/G	69 (39.7)	68 (39.5)	0.90 (0.57–1.41)	0.65
A/A	17 (9.8)	26 (15.1)	0.58 (0.29–1.15)	0.12
G/G vs. A/G-A/A			0.84 (0.55–1.28)	0.41
G/G-A/G vs. A/A			0.59 (0.31–1.13)	0.11
G	245 (70)	224 (65)	1.27 (0.93–1.75)	0.14
A	103 (30)	120 (35)	0.78 (0.57–1.08)	0.14

Abbreviations: CI, confidence interval; OR, odds ratio.

that the ATG16L1 rs2241880 variant may change the binding affinity of several transcription factors, including REST, GATA-1, TFII-I, and ETV4. In addition, rSNPBase³² results showed that ATG16L1 rs2241880 may affect the proximal regulation. Alterations in the transcription binding affinity may be a causative factor contributing to colorectal cancer development. These transcription factors play an important role in several cancers. For example, REST (RE1 Silencing Transcription Factor) is a protein-coding gene that acts as a putative tumor suppressor in different colon cell lines and GATA-1, a member of the GATA transcription factor family, is overexpressed in colorectal cancer, and predicts poor clinical outcome in CRC.^{33,34}

We further investigated the association between ATG5 rs1475270 polymorphism and CRC susceptibility in our studied population. Previously, the association between variations in ATG5 gene and enhanced risk of tumorigenesis was reported in multiple cancers, including breast cancer, melanoma and squamous cell carcinoma.^{35,36} However, we failed to detect any association between ATG5 rs1475270 and the CRC risk in our study. According to HaploReg,³⁰ ORegAnno,³⁷ and PROMO³¹ software, it is suggested that the rs1475270 variant may influence the binding affinity of different transcription factors and proteins, including Dbx2, Hoxb3, XBP-1, SMARCA4, and c-Jun. SMARCA4 acts as a tumor-suppressor gene and is often mutated or silenced in tumors.³⁸

In conclusion, taking into account the frequency and the heavy burden of CRC on the social and economic health system, more attention is needed to determine the susceptible factors that may impact the risk of the disease. Current data suggest that ATG16L1 rs2241880 may impact on CRC risk and support the potential prognostic role of this variant in colorectal cancer based on population. However, our concept about the autophagy process might be limited depending on the tumor conditions and types. The present

TABLE 3 Allele and genotype frequencies of for ATG16L1 rs2241880 and ATG5 rs1475270 in patients with CRC and controls

investigation suffers from limitations such as relatively small sample size. Therefore, confirmation in larger sample size is required. Second, our study is restricted to the Iranian population and cannot be generalized to diverse ethnic populations. So, more studies in different populations are required.

CONFLICT OF INTEREST

The authors declare no conflict of interest in this work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Hossein Sadeghi  <https://orcid.org/0000-0001-9599-6151>

Mohammad-Reza Ghasemi  <https://orcid.org/0000-0002-3183-5495>

Reza Mirfakhraie  <https://orcid.org/0000-0003-1709-8975>

REFERENCES

1. Siegel RL, Miller KD, Fedewa SA, et al. Colorectal cancer statistics, 2017. *CA Cancer J Clin*. 2017;67(3):177–193.
2. Pourhoseingholi MA. Increased burden of colorectal cancer in Asia. *World J Gastrointest Oncol*. 2012;4(4):68.
3. Gandomani HS, Yousefi SM, Aghajani M, et al. Colorectal cancer in the world: incidence, mortality and risk factors. *Biomed Res and Ther*. 2017;4(10):1656–1675.
4. Dolatkah R, Somi MH, Kermani IA, et al. Increased colorectal cancer incidence in Iran: a systematic review and meta-analysis. *BMC Public Health*. 2015;15(1):997.
5. Hagggar FA, Boushey RP. Colorectal cancer epidemiology: incidence, mortality, survival, and risk factors. *Clin Colon Rectal Surg*. 2009;22(04):191–197.

6. Johnson CM, Wei C, Ensor JE, et al. Meta-analyses of colorectal cancer risk factors. *Cancer Causes Control*. 2013;24(6):1207-1222.
7. Vargas AJ, Thompson PA. Diet and nutrient factors in colorectal cancer risk. *Nutr Clin Pract*. 2012;27(5):613-623.
8. Huxley RR, Ansary-Moghaddam A, Clifton P, Czernichow S, Parr CL, Woodward M. The impact of dietary and lifestyle risk factors on risk of colorectal cancer: a quantitative overview of the epidemiological evidence. *Int J Cancer*. 2009;125(1):171-180.
9. Migliore L, Migheli F, Spisni R, Coppedè F. Genetics, cytogenetics, and epigenetics of colorectal cancer. *Biomed Res Int*. 2011;2011:792362.
10. Sameer ASS. Colorectal cancer: molecular mutations and polymorphisms. *Front Oncol*. 2013;3:114.
11. Wu WK, Coffelt SB, Cho CH, et al. The autophagic paradox in cancer therapy. *Oncogene*. 2012;31(8):939-953.
12. Marinković M, Šprung M, Buljubašić M, Novak I. Autophagy modulation in cancer: current knowledge on action and therapy. *Oxid Med Cell Longev*. 2018;2018:8023821.
13. Amaravadi R, Kimmelman AC, White E. Recent insights into the function of autophagy in cancer. *Genes Dev*. 2016;30(17):1913-1930.
14. Burada F, Nicoli ER, Ciurea ME, Uscatu DC, Ioana M, Gheonea DI. Autophagy in colorectal cancer: an important switch from physiology to pathology. *World J Gastrointest Oncol*. 2015;7(11):271-284.
15. Janji B, Berchem G, Chouaib S. Targeting autophagy in the tumor microenvironment: new challenges and opportunities for cancer immunotherapy. *Front Immunol*. 2018;9:887.
16. Mokarram P, Albokashy M, Zarghooni M, et al. New frontiers in the treatment of colorectal cancer: autophagy and the unfolded protein response as promising targets. *Autophagy*. 2017;13(5):781-819.
17. Klionsky DJ, Schulman BA. Dynamic regulation of macroautophagy by distinctive ubiquitin-like proteins. *Nat Struct Mol Biol*. 2014;21(4):336.
18. Sameer AS, Banday MZ, Nissar S. Mutations and polymorphisms: what is the difference? In Aga SS, Banday MZ, Nissar S, eds. *Genetic Polymorphism and Cancer Susceptibility*. Springer; 2021:1-21.
19. Zhang H-F, Qiu L-X, Chen Y, et al. ATG16L1 T300A polymorphism and Crohn's disease susceptibility: evidence from 13,022 cases and 17,532 controls. *Hum Genet*. 2009;125(5-6):627-631.
20. Burada F, Ciurea ME, Nicoli R, et al. ATG16L1 T300A polymorphism is correlated with gastric cancer susceptibility. *Pathol Oncol Res*. 2016;22(2):317-322.
21. Diler SB, Aybuğa F. Association of autophagy gene ATG16L1 polymorphism with human prostate cancer and bladder cancer in turkish population. *Asian Pac J Cancer Prev*. 2018;19(9):2625-2630.
22. Al-Ali R, Fernández-Mateos J, González-Sarmiento R. Association of autophagy gene polymorphisms with lung cancer. *Gene Rep*. 2017;7:74-77.
23. Gaaib JN. Simple salting-out method for genomic DNA extraction from whole blood. *Tikrit J Pure Sci*. 2011;16(2):9-11.
24. Collins A, Ke X. Primer1: primer design web service for tetra-primer ARMS-PCR. *Open Bioinform J*. 2012;6(1):55-58.
25. Sole X, Guino E, Valls J, Iniesta R, Moreno V. SNPStats: a web tool for the analysis of association studies. *Bioinformatics*. 2006;22(15):1928-1929.
26. Cao H, Li Z, Zhou D, et al. ATG16L1 rs2241880 polymorphism predicts unfavorable clinical outcomes for colorectal cancer patients in the Chinese population. *Int J Clin Exp Pathol*. 2016;9(8):8586-8595.
27. Grimm WA, Messer JS, Murphy SF, et al. The Thr300Ala variant in ATG16L1 is associated with improved survival in human colorectal cancer and enhanced production of type I interferon. *Gut*. 2016;65(3):456-464.
28. Nicoli E-R, Dumitrescu T, Uscatu C-D, et al. Determination of autophagy gene ATG16L1 polymorphism in human colorectal cancer. *Rom J Morphol Embryol*. 2014;55(1):57-62.
29. Fernández-Mateos J, Seijas-Tamayo R, Klain JCA, et al. Analysis of autophagy gene polymorphisms in Spanish patients with head and neck squamous cell carcinoma. *Sci Rep*. 2017;7(1):6887.
30. Ward LD, Kellis M. HaploReg v4: systematic mining of putative causal variants, cell types, regulators and target genes for human complex traits and disease. *Nucleic Acids Res*. 2016;44(D1):D877-D881.
31. Messeguer X, Escudero R, Farre D, Nunez O, Martinez J, Alba MM. PROMO: detection of known transcription regulatory elements using species-tailored searches. *Bioinformatics*. 2002;18(2):333-334.
32. Guo L, Du Y, Chang S, Zhang K, Wang J. rSNPBase: a database for curated regulatory SNPs. *Nucleic Acids Res*. 2014;42(D1):D1033-D1039. 10.1093/nar/gkt1167
33. Westbrook TF, Martin ES, Schlabach MR, et al. A genetic screen for candidate tumor suppressors identifies REST. *Cell*. 2005;121(6):837-848.
34. Yu J, Liu M, Liu H, Zhou L. GATA1 promotes colorectal cancer cell proliferation, migration and invasion via activating AKT signaling pathway. *Mol Cell Biochem*. 2019;457(1-2):191-199.
35. Li M, Ma F, Wang J, et al. Genetic polymorphisms of autophagy-related gene 5 (ATG5) rs473543 predict different disease-free survivals of triple-negative breast cancer patients receiving anthracycline-and/or taxane-based adjuvant chemotherapy. *Chin J Cancer*. 2018;37(1):4.
36. White KA, Luo L, Thompson TA, et al. Variants in autophagy-related genes and clinical characteristics in melanoma: a population-based study. *Cancer Med*. 2016;5(11):3336-3345.
37. Lesurf R, Cotto KC, Wang G, et al. Open regulatory annotation C (2016) ORegAnno 3.0: a community-driven resource for curated regulatory annotation. *Nucleic Acids Res*. 44(D1):D126-D132.
38. Guerrero-Martínez JA, Reyes JC. High expression of SMARCA4 or SMARCA2 is frequently associated with an opposite prognosis in cancer. *Sci Rep*. 2018;8(1):1-17.

How to cite this article: Jamali L, Sadeghi H, Ghasemi M-R, et al. Autophagy ATG16L1 rs2241880 impacts the colorectal cancer risk: A case-control study. *J Clin Lab Anal*. 2021;00:e24169. doi:[10.1002/jcla.24169](https://doi.org/10.1002/jcla.24169)