Single nucleotide polymorphism rs4961 in the adducin 1 gene is not associated with gastric cancer or preneoplastic cancer lesions

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Abstract. Gastric cancer (GC) is the fourth most deadly cancer globally. The adducin 1 (ADD1) protein is involved in oncogenic signal transduction pathways in several types of cancer, and the rs4961 variant (c.1378 G>T, p.Gly460Trp) of the *ADD1* gene is associated with salt-sensitive hypertension, renal cell cancer and breast cancer susceptibility; however, it has not been investigated in GC. The aim of the present study was to evaluate the association between the rs4961 variant and the development of GC and preneoplastic gastric lesions (PGLs) in a population from western Mexico. A total of 225 individuals who underwent an endoscopy were evaluated, of which 71 patients had histopathologically diagnosed GC and 53 patients had PGLs, with 101 patients used as controls. The rs4961 variant was genotyped by using PCR and DNA sequencing. The frequency of the mutated homozygous genotype (TT) of the rs4961 variant was <10% in the three evaluated groups, and the frequency of the minor allele (T) was <21% in the GC, PGL and control groups. Genotypic and allelic frequencies were similarly distributed in all of the studied groups (P>0.05). In summary, in the study population, the rs4961 variant was not associated with GC risk; however, its role in other populations and in other types of cancer is worthy of future research.

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Introduction

Gastric cancer (GC) was the fourth most common cancer globally in 2020, and 769,000 people died from GC that year according to GLOBOCAN estimates (1). A high sodium intake explains many cases of GC, and this association can be explained by two important factors: i) Salt strongly irritates the stomach wall and promotes chemical gastric carcinogenesis; and ii) excess salt induces gastric colonization of *Helicobacter pylori* in the stomach, which is a known risk factor for GC (2).

Genetic factors serve an important role in gastric carcinogenesis due to aberrant gene expression, which leads to a malignant phenotype (3). A total of ≥44 GC-related genes have been reported to date (4). Adducin (ADD) family members are involved in oncogenic signal transduction pathways in several types of cancer (5). The *ADD1* gene encodes adducin 1, which is a cytoskeletal protein that is ubiquitously expressed and implicated in the formation of actin-spectrin complexes, actin polymerization and cell signal transduction (6,7). The rs4961 c.1378 G>T p.Gly460Trp variant of the *ADD1* gene located at chromosome 4p16.3 has been associated with salt-sensitive hypertension, and it is reported that ethnic differences and genetic background may be the main causes of salt sensitivity (8-11). This variant is also related to other diseases, such as hemorrhagic stroke, atherosclerosis, myocardial infarction and renal disease (5).

Genetic variation in several genes may account for the increased salt sensitivity of certain individuals (12). Moreover, when regarding the predisposition towards GC, individuals who are not sensitive to salt may ingest larger amounts of salt than necessary or normal when attempting to taste salt in food, thus increasing the likelihood of developing GC. To the best of our knowledge, the association between the rs4961 variant of the *ADD1* gene and the risk of developing GC has not yet been assessed. Therefore, the aim of the present study was to evaluate the association between the rs4961 variant and the development of preneoplastic gastric lesions (PGLs) and GC in a population from western Mexico.

Table I. Demographic data of the three groups studied.

Variable	Gastric cancer (n=71)	P-value	Preneoplastic gastric lesions (n=53)	P-value	Controls (n=101)	P-value
Sex		0.00300[a]		0.73700[b]		0.02600[c]
Female	26 (36.6)		30 (56.6)		60 (59.4)	
Male	45 (63.4)		23 (43.4)		41 (40.6)	
Age, years	59.8±14.8	0.00003[a]	58.9±18.1	0.00090[b]	46.5±23.0	0.38500[c]

Data are presented as n (%) or mean ± standard deviation, and analyzed using the χ^2 or unpaired Student's t-test. [a]Gastric cancer vs. Controls; [b]Preneoplastic gastric lesions vs. Controls; [c]Gastric cancer vs. Preneoplastic gastric lesions.

Table II. Genotypic and allelic frequencies of the *ADD1* rs4961 variant observed in the studied groups.

Frequency	Gastric cancer (n=71)	P-value	Preneoplastic gastric lesions (n=53)	P-value	Controls (n=101)	P-value
Genotype		0.301[a]		0.408[b]		0.705[c]
Wildtype GG	52 (73.2)		35 (66.0)		67 (66.3)	
Heterozygous GT	15 (21.1)		14 (26.4)		31 (30.7)	
Mutated homozygous TT	4 (5.6)		4 (7.5)		3 (3.0)	
Allele		0.732[a]		0.603[b]		0.406[c]
Allele wildtype (G)	119 (83.8)		84 (79.2)		166 (81.7)	
Allele mutated (T)	23 (16.2)		22 (20.8)		36 (18.3)	

Data are presented as n (%) and were analyzed using Fisher's exact test. [a]Gastric cancer vs. Controls; [b]Preneoplastic gastric lesions vs. Controls; [c]Gastric cancer vs. Preneoplastic gastric lesions. ADD1, adducin 1.

Materials and methods

The present study assessed 225 subjects recruited from March 2018 to November 2022 from the gastroenterology services of the National Medical Center of the West, Hospital 110, Hospital 14 and Ambulatory Care Medical Unit 52 of the Mexican Social Security Institute (Guadalajara, Mexico).

The subjects underwent endoscopy as part of their diagnosis, and a biopsy was taken for histopathological analysis (GC, n=71; PGL, n=53; and controls, n=101). Briefly, 5 μm-thick tissue sections were placed on electrocharged slides, and hematoxylin and eosin staining was applied as follows, the electrocharged slides were placed at 55˚C for 15 min, placed in xylol for 5 min and then in decreasing ethanol solutions (100, 90, 70 and 30%), and finally in distilled water. Subsequently, the samples were placed in hematoxylin for 8 min at room temperature, rinsed and placed in an acidic alcohol bath, and rinsed and placed in lithium chloride for 1 min. After which, the samples were rinsed and left for 30 sec in alcohol, and then they were placed in eosin for 1 min at room temperature. The sections were rinsed and dehydrated in solutions of increasing concentrations of ethanol, and subsequently placed in xylol for 5 min. Finally, the sections were covered with resin and coverslips, and allowed to dry for 8 h before observation under an optical microscope. All of the subjects in the GC group were adults, and the ages in the PGL and control groups ranged from 2-89 years. Diagnoses was made using a histopathological assessment of the biopsies by a pathologist, and based on Lauren's classification (13), with GC including diffuse and intestinal types and PGL involving atrophic gastritis and intestinal metaplasia. The control group consisted of subjects with non-atrophic gastritis (Fig. S1). The inclusion criteria was as follows: i) Individuals who would undergo an endoscopy study as part of their diagnosis; ii) those who were not undergoing any treatment for chronic gastritis or gastric cancer; and iii) those who agreed to participate in the present study and signed a letter of informed consent. Individuals whose gastric tissue or DNA samples did not have sufficient quality or quantity for analysis were excluded from the present study.

DNA was extracted from peripheral blood leukocytes using the salting out method, as described by Miller *et al* (14). To identify the rs4961 G>T variant of the *ADD1* gene, a 415 bp DNA fragment including the region of interest was amplified using PCR. The following primers were designed by Oligo software (version 6.0; https://oligo.net/oligo_updates.htm): Forward, 5'-GGGCTACAGAACTGGCTACC-3' and reverse, 5'-GCCTCCGAAGCCCCAGCTACCCA-3'. The reaction was performed under the following conditions: 100 ng of genomic DNA, 5 pM of each primer, 0.5 U of Taq polymerase (Invitrogen™; Thermo Fisher Scientific, Inc.), 1X PCR buffer, 1.5 mM $MgCl_2$ and 2 mM deoxynucleotide triphosphate mix (dNTP Set; Vivantis Technologies Sdn. Bhd.). The PCR conditions were as follows: 94˚C for 5 min; 30 cycles of 94˚C

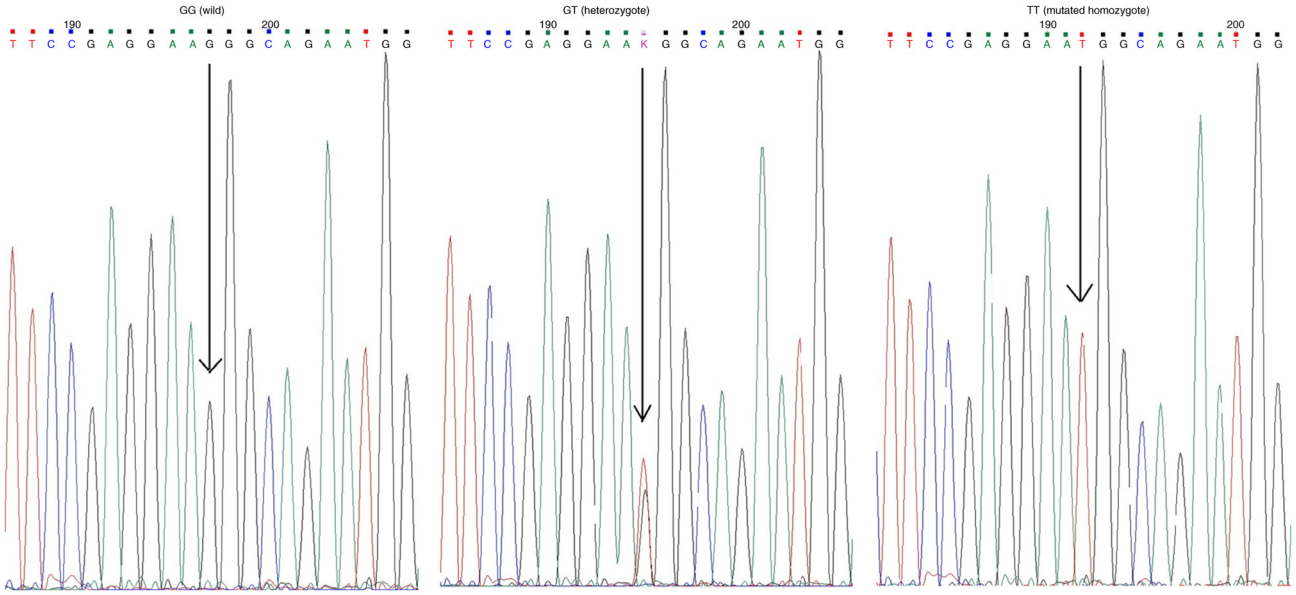

Figure 1. Genotypes of the rs4961 variant of the adducin 1 gene, obtained by Sanger capillary sequencing (arrows indicate nucleotide changes).

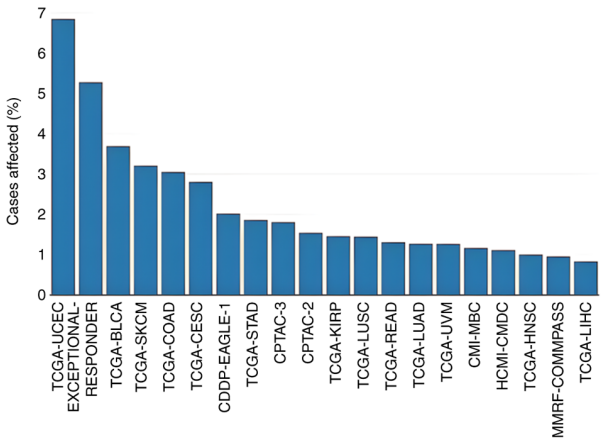

Figure 2. Proportion of cancer cases in each dataset in the Genomic Data Commons portal where there is a mutation (except copy number variants) in the adducin 1 gene. BLCA, bladder urothelial carcinoma; CDDP-EAGLE-1, CDDP Integrative Analysis of Lung Adenocarcinoma (Phase 2); CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CMI-MBC, count Me In (CMI): The Metastatic Breast Cancer (MBC) Project; COAD, colon adenocarcinoma; CPTAC-2, CPTAC-Breast, Colon, Ovary; CPTAC-3, CPTAC-Brain, Head and Neck, Kidney, Lung, Pancreas, uterus; HCMI-CMDC, NCI Cancer Model Development for the Human Cancer Model Initiative; HNSC, head and neck squamous cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MMRF-COMMPASS, Multiple Myeloma CoMMpass Study; READ, rectum adenocarcinoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TCGA, The Cancer Genome Atlas; UCEC, uterine corpus endometrial carcinoma; UVM, uveal melanoma.

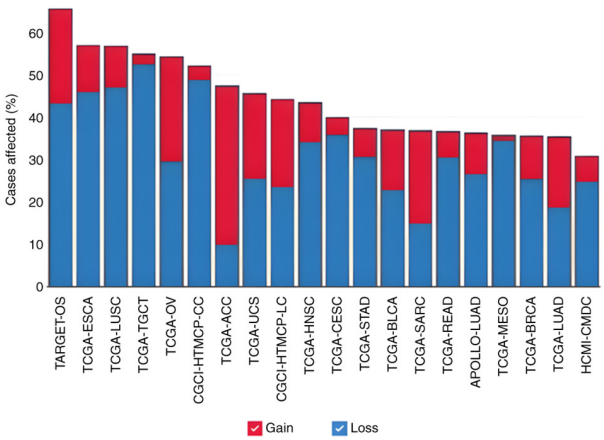

Figure 3. Proportion of cancer cases in each dataset in the Genomic Data Commons portal where there are copy number variants mutations in adducin 1 gene. ACC, adrenocortical carcinoma; APOLLO-LUAD, APOLLO1: Proteogenomic characterization of lung adenocarcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CGCI-HTMCP-CC, HIV+ Tumor Molecular Characterization Project-Cervical Cancer; CGCI-HTMCP-LC, HIV+ Tumor Molecular Characterization Project-Lung Cancer; ESCA, esophageal carcinoma; HCMI-CMDC, NCI Cancer Model Development for the Human Cancer Model Initiative; HNSC, head and neck squamous cell carcinoma; LUSC, lung squamous cell carcinoma; MESO, mesotelioma; OV, ovarian serous cystadenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; STAD, stomach adenocarcinoma; TARGET-OS, TARGET osteosarcoma; TCGA, The Cancer Genome Atlas; TGCT, testicular germ cell tumors; UCS, uterine carcinosarcoma.

for 1 min, 62˚C for 1 min, and 72˚C for 1 min; then 72˚C for 5 min. This was performed by using a 2720 Thermal Cycler (Applied Biosystems™; Thermo Fisher Scientific, Inc.). The resulting fragment was purified using ExoSAP-IT™ PCR Product Cleanup Reagent (Applied Biosystems; Thermo Fisher Scientific, Inc.) and Sanger capillary sequencing was performed using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the SeqStudio Genetic Analyser (Applied Biosystems; Thermo Fisher Scientific, Inc.) and analyzed using Chromas DNA Sequencing Software (https://technelysium.com.au/wp/chromas/).

The allelic and genotypic frequencies were obtained by direct counting, and comparisons between groups were analyzed using Fisher's exact test using the PASW Statistic Base 18 software (SPSS, Inc.). Other variables, such as sex and

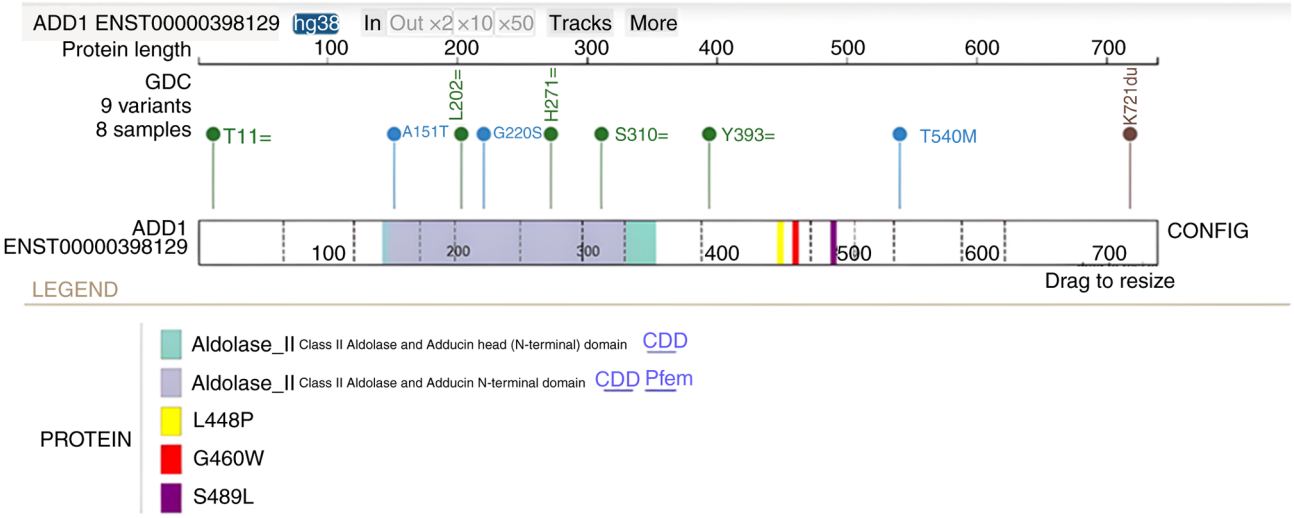

Figure 4. In the gastric cancer cohort of the GDC portal (n=824), 8 patients had 9 ADD1 mutations (not including copy number variants), presented as green circles (silent), blue circles (missense) and brown circles (stop-gain). Other relevant mutations in other types of cancers are shown as a yellow bar (L448P; skin cancer), red bar (G460W; the present study) and purple bar (S489L; colon and mouth cancer). GDC, Genomic Data Commons; ADD1, adducin 1.

age, were analyzed using the χ^2 and unpaired Student's t-tests. P<0.05 was considered to indicate a statistically significant difference.

In addition, a cohort from the Genomic Data Commons (GDC) portal (https://portal.gdc.cancer.gov/; v.39.0) was obtained, which included patients for whom the stomach was the primary cancer site (n=824). The datasets (appears as 'projects' in the GDC portal) included were EXCEPTIONAL_RESPONDERS-ER, FM-AD, HCMI-CMDC, MATCH-S1, MATCH-Z1D, TARGET-NBL, TCGA-DLBC, TCGA-ESCA, TCGA-SARC and TCGA-STAD. These datasets (projects) belong to the following programs: EXCEPTIONAL_RESPONDERS, FM, HCMI, MATCH, TARGET and TCGA. Mutations in the *ADD1* gene and demographic data in the cohort were searched for using ProteinPaint, Mutation Frequency and OncoMatrix analysis tools, which are all freely available at the GDC portal (https://portal.gdc.cancer.gov/).

Results

The demographic data of the three groups are reported in Table I. The GC group had a significantly greater proportion of males (63%) than females (37%) compared with the control group (41% vs. 59%, respectively; P=0.003); however, the PGL group had a similar proportion compared with the control group (P=0.737) (Table I). Furthermore, the odds ratio (OR) for male sex was evaluated, and the results demonstrated an increased risk of GC in males compared with that in controls, with an OR of 2.5 and a 95% confidence interval (CI) of 1.35-4.73 (P=0.003; Table I). Conversely, mean age was significantly higher in the GC group (59.8 years) than in the PGL and control groups (58.9 and 46.5 years, respectively; P<0.05; Table I).

The frequency of the mutated homozygous genotype (TT) of the rs4961 variant was <10% in the three evaluated groups, and the frequency of the minor allele (T) was <21% in the GC, PGL and control groups (Table II). The allele and genotype frequencies of rs4961 polymorphism were in the Hardy-Weinberg equilibrium (P>0.05; data not shown).

The distributions of allelic and genotypic frequencies of rs4961 were compared between the three groups; however, no differences were observed in genotypic or allelic frequencies between GCs and controls or between PGLs and controls (P>0.05; Table II). The wild-type, heterozygous, and homozygous mutated genotypes observed by Sanger sequencing are shown in Fig. 1.

Furthermore, data from the GDC portal revealed that the *ADD1* gene was altered in 4,103 patients with cancer affected by 3,975 copy number variation (CNV) events (duplications + deletions) across 47 datasets (Fig. 2). In addition, 182 patients were affected by 189 other non-CNV mutations in the *ADD1* gene across 27 projects (Fig. 3). Subsequently, analysis of the GC cohort (n=824) demonstrated that there were 8 patients with 5 silent, 3 missense and 1 stop-gain mutations in *ADD1* (Fig. 4). Notably, L448P and S489L are two likely damaging mutations (Polyphen score >0.99; https://www.ensembl.org/info/genome/variation/prediction/protein_function.html) and both mutations are in proximity to rs4961 (Fig. 4). The L448P mutation was found to be present in two Caucasian male patients with skin cancer and the S489L mutation was present in two Caucasian male patients with colon and mouth cancer in the database. Within the same cohort, but excluding CNVs (female, n=9 and male, n=28), the most commonly mutated genes were Mucin 16, AT-rich interactive domain-containing protein 1A and tumor protein 53 (Fig. 5). CNVs in the GC cohort are presented in Fig. 6.

Discussion

The rs4961 c.1378 G>T, p.Gly460Trp variant of the *ADD1* gene has been associated with salt-sensitive hypertension and renal cell cancer risk and is a candidate for breast cancer susceptibility (15,16). To the best of our knowledge, the role of the rs4961 variant in GC risk has not been previously assessed.

In the present study, the rs4961 variant of the *ADD1* gene was evaluated in relation to GC risk, and the results excluded the rs4961 variant as being a risk factor for gastric carcinogenesis in the Mexican population. However, additional studies

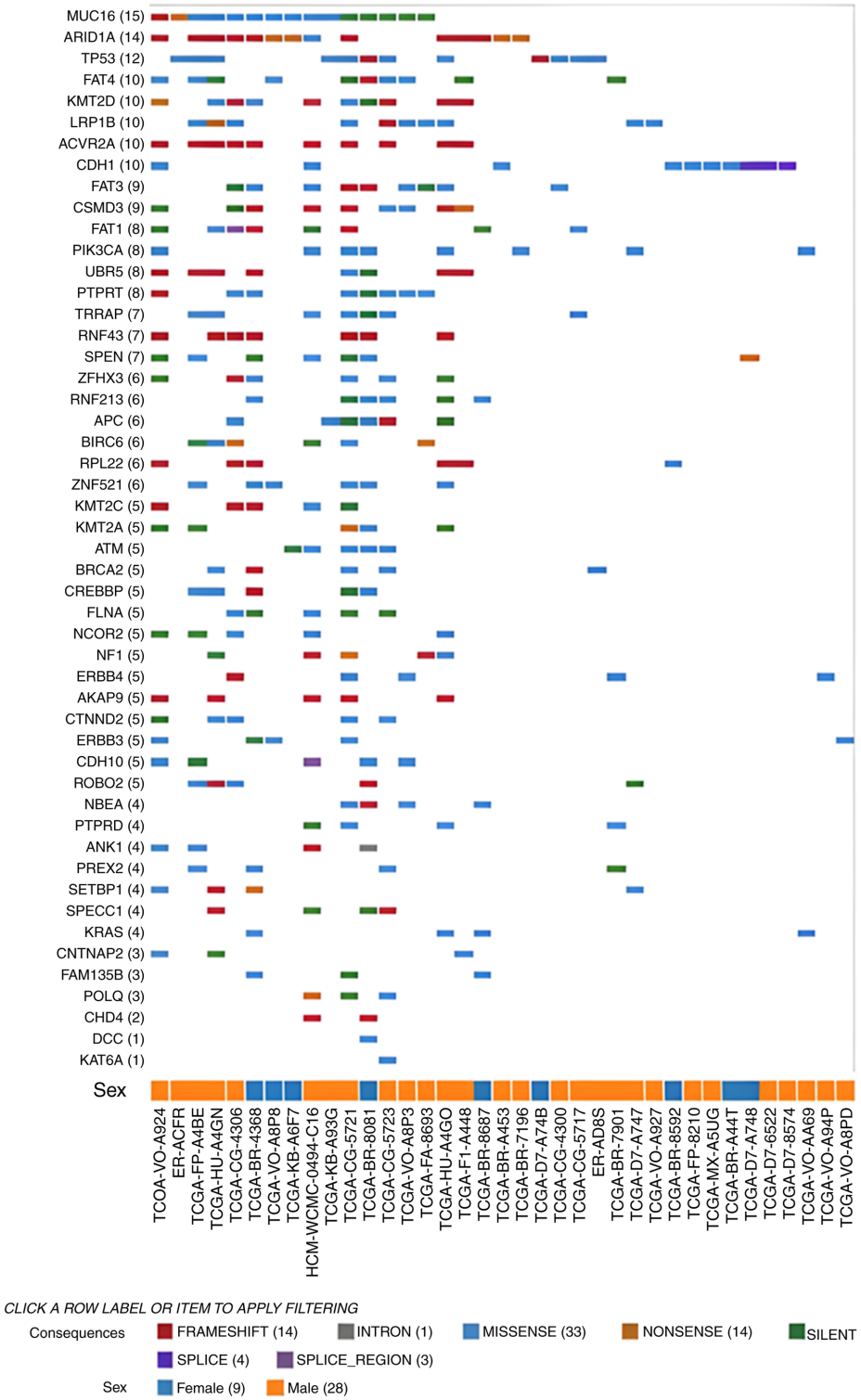

Figure 5. Mutations (excluding copy number variants) found in the gastric cancer cohort (n=824) of the Genomic Data Commons portal. Each row is a gene with the highest mutation frequencies from top to bottom. Each column refers to a patient: Males (orange squares) and females (blue squares). The codes at the bottom of the figure refers to the unique ID of each patient. *ACVR2A*, activin A receptor type 2A; *AKAP9*, A-kinase anchoring protein 9; *ANK1*, ankyrin 1; *APC*, APC regulator of WNT signaling pathway; *ARID1A*, AT-rich interaction domain 1A; *ATM*, ATM serine/threonine kinase; *BIRC6*, baculoviral IAP repeat containing 6; *BRCA2*, BRCA2 DNA repair associated; *CDH1*, cadherin 1; *CDH10*, cadherin 10; *CHD4*, chromodomain helicase DNA binding protein 4; *CNTNAP2*, contactin associated protein 2; *CREBBP*, CREB binding protein; *CSMD3*, CUB and Sushi multiple domains 3; *CTNND2*, catenin delta 2; *DCC*, DCC netrin 1 receptor; *ERBB4*, erb-b2 receptor tyrosine kinase 4; *FAM135B*, family with sequence similarity 135 member B; *FAT1*, FAT atypical cadherin 1; *FAT3*, FAT atypical cadherin 4; *FAT4*, FAT atypical cadherin 4; *FLNA*, filamin A; *KAT6A*, lysine acetyltransferase 6A; *KMT2A*, lysine methyltransferase 2A; *KMT2C*, lysine methyltransferase 2C; *KMT2D*, lysine methyltransferase 2D; *KRAS*, KRAS proto-oncogene, GTPase; *LRP1B*, LDL receptor related protein 1B; *MUC16*, mucin 16; *NBEA*, neurobeachin; *NCOR2*, nuclear receptor corepressor 2; *NF1*, neurofibromin 1; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; *POLQ*, DNA polymerase theta; *PTPRD*, protein tyrosine phosphatase receptor type D; *PTPRT*, protein tyrosine phosphatase receptor type T; *PREX2*, phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 2; *ROBO2*, roundabout guidance receptor 2; *RPL22*, ribosomal protein L22; *RNF43*, ring finger protein 43; *RNF213*, ring finger protein 213; *SETBP1*, SET binding protein 1; *SPECC1*, sperm antigen with calponin homology and coiled-coil domains; *SPEN*, spen family transcriptional repressor; *TP53*, tumor protein p53; *TRRAP*, transformation/transcription domain associated protein; *UBR5*, ubiquitin protein ligase E3 component n-recognin 5; *ZFHX3*, zinc finger homeobox 3; *ZNF521*, zinc finger protein 521.

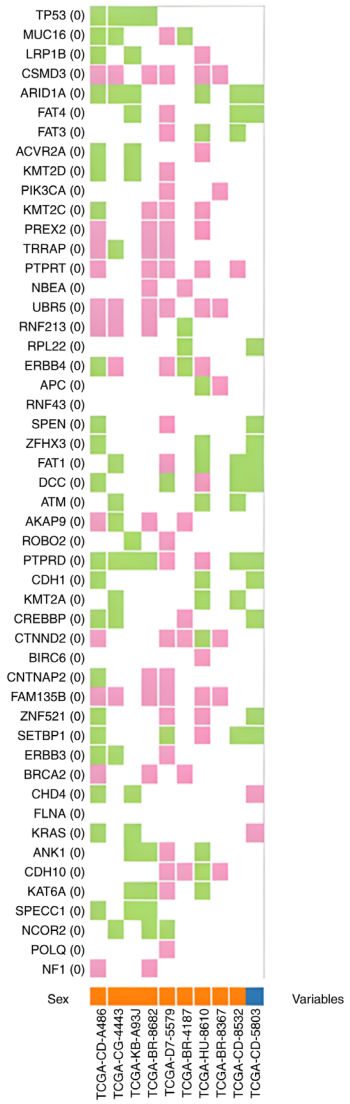

Figure 6. Copy number variants found in the gastric cancer cohort (n=824) of the Genomic Data Commons portal, with copy number gains (pink) and losses (green). Each column refers to each patient: Males (orange squares) and females (blue square).The codes at the bottom of the figure refers to the unique ID of each patient. *ACVR2A*, activin A receptor type 2A; *AKAP9*, A-kinase anchoring protein 9; *ANK1*, ankyrin 1; *APC*, APC regulator of WNT signaling pathway; *ARID1A*, AT-rich interaction domain 1A; *ATM*, ATM serine/threonine kinase; *BIRC6*, baculoviral IAP repeat containing 6; *BRCA2*, BRCA2 DNA repair associated; *CDH1*, cadherin 1; *CDH10*, cadherin 10; *CHD4*, chromodomain helicase DNA binding protein 4; *CNTNAP2*, contactin associated protein 2; *CREBBP*, CREB binding protein; *CSMD3*, CUB and Sushi multiple domains 3; *CTNND2*, catenin delta 2; *DCC*, DCC netrin 1 receptor; *ERBB4*, erb-b2 receptor tyrosine kinase 4; *FAM135B*, family with sequence similarity 135 member B; *FAT1*, FAT atypical cadherin 1; *FAT3*, FAT atypical cadherin 4; *FAT4*, FAT atypical cadherin 4; *FLNA*, filamin A; *KAT6A*, lysine acetyltransferase 6A; *KMT2A*, lysine methyltransferase 2A; *KMT2C*, lysine methyltransferase 2C; *KMT2D*, lysine methyltransferase 2D; *KRAS*, KRAS proto-oncogene, GTPase; *LRP1B*, LDL receptor related protein 1B; *MUC16*, mucin 16; *NBEA*, neurobeachin; *NCOR2*, nuclear receptor corepressor 2; *NF1*, neurofibromin 1; *PIK3CA*, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; *POLQ*, DNA polymerase theta; *PTPRD*, protein tyrosine phosphatase receptor type D; *PTPRT*, protein tyrosine phosphatase receptor type T; *PREX2*, phosphatidylinositol-3,4,5-trisphosphate dependent Rac exchange factor 2; *ROBO2*, roundabout guidance receptor 2; *RPL22*, ribosomal protein L22; *RNF43*, ring finger protein 43; *RNF213*, ring finger protein 213; *SETBP1*, SET binding protein 1; *SPECC1*, sperm antigen with calponin homology and coiled-coil domains; *SPEN*, spen family transcriptional repressor; *TP53*, tumor protein p53; *TRRAP*, transformation/transcription domain associated protein; *UBR5*, ubiquitin protein ligase E3 component n-recognin 5; *ZFHX3*, zinc finger homeobox 3; *ZNF521*, zinc finger protein 521.

are needed to determine the role of this variant in GC in this and other populations, as well as the relationship between the *ADD1* gene and cancer.

Notably, interethnic differences may explain the observed differences in the effect of the rs4961 variant. For instance, a previous study reported that in patients with hypertension and coronary artery disease, Black patients and *ADD1* variant carriers (GT or TT) were at greater risk of a primary outcome event than those with wild-type homozygotes [GG; adjusted hazard ratio (HR), 2.62; 95% CI, 1.23-5.58; P=0.012), with an 8-fold increase in the risk of death and a similar trend in White (HR, 1.24; 95% CI, 0.90-1.71) and Hispanic patients (adjusted HR, 1.43; 95% CI, 0.86-2.39); however, the difference was not-significant and had a smaller magnitude of effect (17).

In a previous study, the *ADD1* rs4961 variant was reported to be related to cancer renal cell risk, with an HR of 1.24 (95% CI, 1.01-1.53) for the GT + TT vs. GG genotype, yet these results were not statistically significant after adjustments for multiple testing (15). Additionally, the variant rs4961 has been reported to be a candidate for breast cancer susceptibility (16). Although there is no direct evidence of the role of *ADD1* in breast cancer progression and tumorigenesis, variants of this gene, such as rs4961 and rs4963, have been reported to occur more frequently in patients with higher-grade malignant breast tumors (III vs. II) (18). Hypertension is also a common comorbidity in patients with breast cancer and is associated with a poor prognosis. Li *et al* (18) reported that 77% of patients with grade III breast cancer carries both rs4961 and rs4963 variants, thus indicating an increased risk of developing hypertension compared with 16% of patients with grade III breast cancer.

Furthermore, the present study demonstrated that male sex confers a significantly increased risk of GC, with an OR of 2.5 (95% CI, 1.35-4.73; P=0.003). Male sex is a well-known risk factor for GC and other types of cancer (19).

Nonetheless, the present study had several limitations, such as the small sample size of patients with GC who were included in the analysis. It is therefore important to consider matching samples by age and sex in future studies. A further limitation of the present study is that the clinicopathological characteristics of patients with GC were not collected, such as tumor size, lymph nodes and tumor infiltration, therefore, it is difficult to specify the relationship of the rs4961 variant of the *ADD1* gene with these variables, as well as with the prognosis of the patients.

In conclusion, in the present population, the *ADD1* rs4961 variant was not associated with GC risk; however, its role in other populations and in other types of cancer is worthy of future research.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

MYAF and KRP performed the PCR and DNA sequencing experiments, contributed to the acquisition, analysis and interpretation of the data and were the major contributors to the writing of the manuscript. SRDLTG contributed to the analysis and interpretation of the data. FJPD, MTMT and EPMDO contributed to the acquisition, analysis and interpretation of data, and manuscript drafting or critical revisions of the intellectual content. JYSL contributed to the conception and design of the study, as well as analysis and interpretation of the data and approval of the final manuscript version to be published. JYSL also agreed to be accountable for all aspects of the work so that any questions relating to research integrity or scientific accuracy in any part of the study could be appropriately investigated and resolved. FJPD and JYSL confirmed the authenticity of all of the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the local Committee of Health Research and Ethics of the Western Biomedical Research Center, Mexican Social Security Institute (Guadalajara, Mexico; approval no. R-2023-1305-009). As the research involved human subjects, written informed consent was obtained from all of the participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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