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Research paper

Single nucleotide polymorphism in *PTEN*-Long gene: A risk factor in chronic myeloid leukemia

C. Ferri[a,*], N. Weich[b,d], L. Gutiérrez[b], C. De Brasi[b], M.R. Bengió[c], P. Zapata[a], A. Fundia[b,1], I. Larripa[b,1]

[a] Laboratorio de Biotecnología Molecular, Instituto de Biotecnología Misiones (InBioMis), Facultad de Ciencias Exactas Químicas y Naturales (FCEQyN), Universidad Nacional de Misiones (UNaM), Ruta Nacional N°12 Km 7.5, Miguel Lanús, 3304 Posadas, Misiones, Argentina
[b] Laboratorio de Genética Hematológica, IMEX, CONICET-Academia Nacional de Medicina, Buenos Aires, Argentina
[c] Departamento de Hemato-oncología, Academia Nacional de Medicina, Buenos Aires, Argentina
[d] Miller School of Medicine, University of Miami, Miami, FL, United States

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ABSTRACT

The *BCR-ABL1* oncogene is associated with chronic myeloid leukemia (CML) pathogenesis, but the molecular mechanisms that initiate leukemogenesis are still unclear. Cancer pathogenesis has been associated with genetic alterations that may lead to inactivation of tumor suppressor genes. Phosphatase and tensin homolog (*PTEN*) is frequently deleted or inactivated in various tumors. A recently discovered variant of PTEN, PTEN-Long (PTEN-L), results from an alternative translation initiation site located upstream of the canonic AUG and generates a protein of 576 amino acids instead the expected protein of 403 amino acids. A 16 bp perfect palindromic motif centered on the *PTEN-L* CUG^{513} start codon is required for translation initiation. A single nucleotide polymorphism (SNP) of *PTEN-L* gene rs12573787 is located on the first exon respect to the CUG initiation site. In this case-control study we evaluated the association of genetic variants in *PTEN-L* with CML risk and therapy response in the Argentine population. The allele A of SNP rs12573787 was found to be associated with CML risk OR (95% CI) 1.71 (1.11–2.63) p = 0.016, which resulted consistent by multivariate analysis adjusted by gender and age. According to previous evidence that CML is more frequent in males, we found that the genetic risk of CML was confined to this gender. Unexpectedly, we also found this association confined to CML patients older than 45 years old. To our knowledge, this is the first time that *PTEN-L* rs1257378 was studied in CML suggesting that the variant A allele is a risk factor for CML development but, no association with the failure to TKIs treatment was found.

1. Introduction

The *BCR-ABL1* oncogene is associated with CML pathogenesis, but the molecular mechanisms that initiate leukemogenesis are still unclear (He et al., 2014; Soverini et al., 2015). CML treatment is based on tyrosine kinase inhibitors (TKIs) that block the chimeric oncoprotein ($p210^{BCR-ABL1}$) (Rychter et al., 2017). Imatinib is still prevalently used as the first-line treatment for newly diagnosed CML patients. Second and third generation TKIs offer higher rates of molecular response compared with standard dose of first generation TKIs (Shanmuganathan et al., 2017). However, approximately 20–30% of treated patients will eventually develop resistance to imatinib (Quintás-Cardama et al., 2009). Moreover, some patients are refractory to all available TKIs (Huang et al., 2016). The best characterized mechanism of resistance is point mutations within the *BCR-ABL1* kinase domain (Gorre et al., 2001; Branford et al., 2003). Different *BCR-ABL1* point mutations generate different specific levels of resistance but the absences of *BCR-ABL1* mutations do not exclude resistance to treatment (Khorashad et al., 2006). TKI resistance has also been related to other mechanisms including clonal chromosomal evolution, *BCR-ABL1* amplification,

Abbreviations: CML, chronic myeloid leukemia; PTEN, phosphatase and tensin homolog; PTEN-L, PTEN-Long; SNP, single nucleotide polymorphism; TKIs, tyrosine kinase inhibitors; qRT, quantitative real time; ARMS-PCR, amplification-refractory mutation system; OR, odds ratios; HWE, Hardy-Weinberg equilibrium; bp, base pair(s)

* Corresponding author at: Laboratorio de Biotecnología Molecular, INBIOMIS – UNaM, Ruta Nacional N°12 Km 7.5, Miguel Lanús, 3304 Posadas, Misiones, Argentina.
E-mail addresses: clnafer@yahoo.com.ar, cristianferri02@gmail.com (C. Ferri).
[1] These authors contributed equally as last authors to this work.

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pharmacogenomic variations, or activation of signaling pathways (La Rosée and Deininger, 2010).

Cancer pathogenesis has been associated with genetic alterations that may conduce to inactivation of tumor suppressor genes. Among these, phosphatase and tensin homolog (*PTEN*) is frequently deleted or inactivated in various tumors suggesting that the loss of a single *PTEN* allele is sufficient to drive tumorigenesis (Berger et al., 2011). *PTEN* gene is located on chromosome 10q23.3 and since it was identified as an important tumor suppressor, it was studied in many human cancers (Li and Sun, 1997; Pulido, 2015). The major biological activity of *PTEN* relies on desphosphorylation of phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P3] to PIP2. PTEN is mostly involved in CML pathogenesis through non genomic loss of function mechanism (Panuzzo et al., 2014). Additionally, *PTEN* is downregulated by *BCR-ABL1* in CML stem cells and its deletion causes acceleration of the disease (Carpten et al., 2007; Peng et al., 2010). We have reported that *LYN/PTEN* ratio showed a direct significant correlation with the level of *BCR-ABL1* transcripts indicating that a high *LYN/PTEN* ratio is related to disease progression (Ferri et al., 2014). A recently discovered variant of PTEN, PTEN-Long (PTEN-L), results from an alternative translation initiation site located upstream of the canonic AUG and generates a protein of 576 amino acids instead the traditional protein of 403 amino acids. PTEN-L exhibit additional functions and has the particularity of being secreted and internalized by other cells. Exogenous PTEN-L antagonizes the PI3K signaling and induces tumor cell death (Hopkins et al., 2013; Hopkins et al., 2014). Translation of *PTEN-L* is initiated from a CUG codon upstream of an in-frame with the coding region of canonical *PTEN*. A 16 bp perfect palindromic motif centered on the *PTEN* CUG^{513} start codon (CCCGCUCCUGGAGCGGG) is required for translation initiation (Liang et al., 2014). Since palindromic regions were highlighted as cause of human disease (Smith, 2008), sequence variants in these regions may achieve great relevance. A single nucleotide polymorphism (SNP) of *PTEN-L* gene (c.10G > A, rs12573787, Gly to Arg) is located on the first exon respect to the CUG initiation site (Wang et al., 2012; Liang et al., 2014). We hypothesize that common genetic polymorphisms, such as SNPs, may modulate the function of *PTEN-L* gene, leading to cancer predisposition or altered treatment responses. However, scarce studies have investigated the role of this SNP in cancer risk and/or its clinical outcome (Xie et al., 2011; Wang et al., 2012). To our knowledge, the influence of genetic variations on *PTEN-L* has not been evaluated in patients with CML so far. The aim of this study was to evaluate the associations between and the SNP rs12573787 genotypes with CML risk and resistance to treatment.

2. Materials and methods

2.1. Study populations

Peripheral blood samples were obtained from 102 patients (55 females and 47 males; mean age 52 ± 1.67; range 18–82 years) diagnosed with CML and under different TKIs treatment (imatinib, dasatinib, nilotinib and ponatinib) with a mean of follow up of 69.97 months. The incorporation of CML patients was voluntary. The inclusion criteria were: 1) Adults (≥18 years) of both sexes with normal cognitive function, 2) diagnosis of CML was determined by clinical and hematologic evaluation, cytogenetic and molecular studies, 3) patients must have been treated with TKI for at least 18 months before study entry and, 4) patients gave the informed consent accepting to enter into the study. Patients were excluded in the following situations: 1) Patients younger than 18 years, 2) mental or psychiatric illness, 3) patients not treated with TKI.

Additionally, 116 sex and age matched unrelated healthy individuals (60 females and 56 males; mean age 48; range 18–83 years) without medical history of leukemia or other chronic diseases, were analyzed. All individuals provided their informed consent according to institutional guidelines. The study was approved by the Institutional Ethical Committee and complies with the International Declaration of Helsinki.

2.2. Patients follow up

European LeukemiaNet recommendations for the management of CML were followed. Accordingly, patients classified as responders reached the hematologic, cytogenetic and molecular response in the time stipulated by the European LeukemiaNet. Patients who did not reach any response or have been lost it were considered resistant to TKI treatment (Baccarani et al., 2013). In particular, 43 patients in chronic phase were in major molecular response or better (31 with imatinib, 6 with nilotinib and 6 with dasatinib) were recruited during their follow-up studies. The remaining 59 patients were catalogued as resistant to TKIs (9 imatinib, 24 nilotinib, 25 dasatinib and 1 ponatinib). These patients with treatment failure were recruited from a research study performed in the National Academy of Medicine from Buenos Aires.

Quantitative real time (qRT)-PCR assay was carried out to measure *BCR-ABL1* transcripts using a Rotor-Gene PCR Cycler (Qiagen®). *BCR–ABL1/ABL1* ratio was determined using the MolecularMD® *BCR–ABL1*^{IS} MR Assay™ following the manufacturer's instructions, according to log reduction in the international scale. Mutational analysis of *ABL1* kinase domain was carried out in 59 resistance patients by direct sequencing. A fragment of 1313 bp of *BCR–ABL1* rearrangement was first amplified followed by a nested PCR that amplifies 586 bp corresponding to the kinase domain of *ABL1* (exons 4–7 covering amino acids 228–423) (Ferri et al., 2013). Sanger sequencing were performed on the 586 bp PCR products, previously purified using GFX columns (GE Healthcare). Sequences were evaluated using the Mutation Surveyor program (SoftGenetics, State College, PA).

2.3. Nucleic acid extraction

Total peripheral blood leukocytes from all cases and control subjects were used for DNA and RNA isolation. Genomic DNA and RNA were extracted using TRIreagent® (Sigma) according to the manufactures' instructions. DNA integrity was checked both in ethidium bromide-stained 1% agarose gel electrophoresis and quantified in a spectrophotometer (GeneQuant pro). RNA concentration was determined by measuring absorbance at 260 nm.

2.4. Analysis of PTEN-long gene

First, mutation profile of *PTEN-L* was analyzed in 25 resistant patients by direct DNA sequencing. An 1878 bp of *PTEN-L* gene was amplified (the analyzed region includes the 173 amino acids prior to the initiation CUG). Primer sequences were: Forward: 5′-TCGGCTGAGAG CTTTCATTT-3′ and reverse: 5′-CATCCGTCTACTCCCACGTT-3′. The products were purified using GFX columns (GE Healthcare); Sanger sequenced and evaluated using the Mutation Surveyor program (SoftGenetics, State College, PA).

Genotyping of *PTEN-L* rs12573787 was performed by amplification-refractory mutation system (ARMS-PCR). Primers were designed using Primer3 Program V.4.0 and their sequences were as follow:

Forward-1: 5′-CACCTCCCGCTCCTGGAGTGGA-3′
Forward-2: 5′-CACCTCCCGCTCCTGGAGTGGG-3′
Reverse: 5′-AGAGACCAACTCTCCGGCGTTC-3′.

Two reactions mixtures were performed separately, one using the combination of the primers Forward-1 and Reverse for the A allele (underlined) while, Forward-2 and Reverse were used for the G allele (underlined). In each PCR reaction, three DNA samples from individuals with known genotypes (heterozygous A/G, a homozygous A/A and homozygous G/G) were used as controls. The amplification reaction was done with 50 ng of genomic DNA and 0.4 μM of each primer.

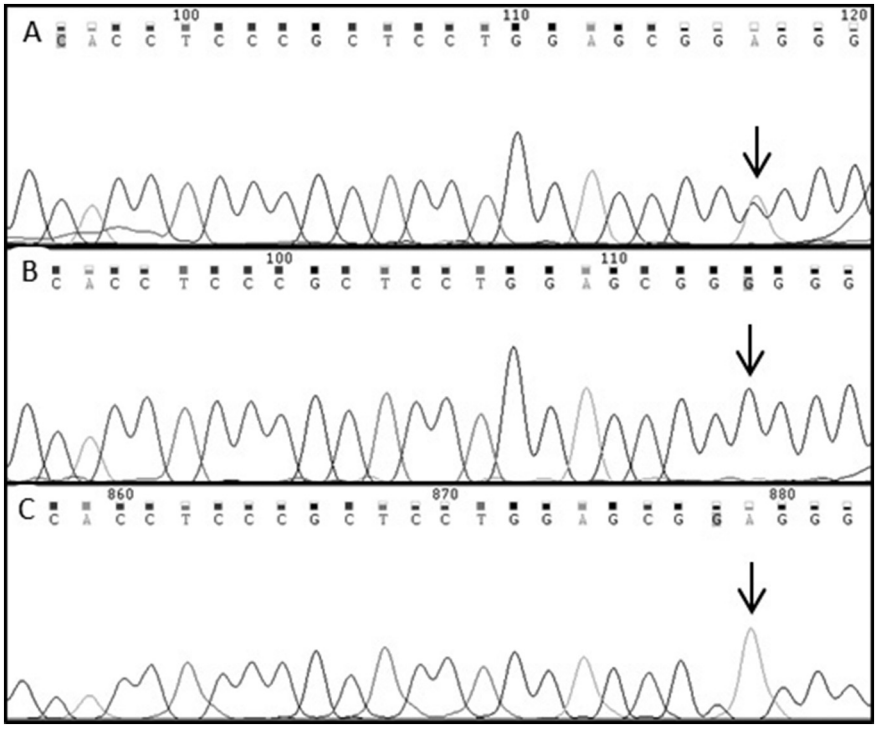

Fig. 1. Genotype patterns for *PTEN-L* rs12573787: (A) Heterozygous G/A; (B) homozygous G/G; (C) homozygous A/A. Arrows indicate the SNP position.

Cycling conditions were 35 cycles of 35 s at 95 °C, 35 s at 60 °C and 40 s at 72 °C. The 129 bp PCR products (G or A) were analyzed by agarose gel electrophoresis (2.5%).

2.5. Statistical analysis

Statistical analyses were performed using SPSS statistical package (version 15.0) (IBM, SPSS Inc., Chicago, USA) and SNP STAT an online tool (Sole et al., 2006). The associations between PTEN-L rs12573787 and CML were performed by multivariate logistic regression analysis. Odds ratios (OR) and their corresponding 95% confidence intervals (CI 95%) were calculated. Hardy-Weinberg equilibrium (HWE) was tested using a goodness-of-fit Chi-square test. Standard genetic models (additive, recessive and dominant) for disease penetrance were evaluated using SNP STAT. All statistical tests were two-sided and values of $p < 0.05$ were considered statistically significant.

3. Results

The presence of *PTEN-L* rs12573787 polymorphism was corroborated by direct sequencing in 25 cases (Fig. 1); the remaining samples were analyzed by ARMS-PCR.

Genotype and allele frequencies distributions for *PTEN-L* rs12573787 in cases and controls are shown in Table 1. No significant deviation from HWE was found in patients ($p = 0.25$) or controls ($p = 0.78$). Comparison of allele frequencies revealed that the A allele was significantly increased in patients (31%) compared to controls (21%) ($p = 0.016$) being a risk factor for CML OR (95% CI) 1.71 (1.11–2.63). Multivariate analysis adjusted for sex and age for various genetic models demonstrated that variant genotypes were significantly associated with the increased risk of CML according to the dominant model OR 1.85 (1.07–3.21) $p = 0.027$ and additive model OR 1.69 (1.05–2.70) $p = 0.028$.

We further conducted the stratification analyses by sex and age to detect whether these potential confounders played roles in the CML risk (Tables 2 and 3). Two age strata (> 45 and ≤45 years old) were defined according to the median age at diagnosis of CML in the Latin American population (Pagnano et al., 2015). The increased risk associated with rs12573787 was significant in the older group (> 45 years) OR 2.16 (1.07–4.35) $p = 0.036$, and in males OR 2.77 (1.23–6.20)

Table 1
Association study between *PTEN-L* rs12573787 SNP and CML risk.

	Genotype	Controls N (%)	Patients N (%)	OR (95% CI)	p-Value
Alleles[a]	G	183 (79)	140 (69)	1 (ref.)	
	A	49 (21)	64 (31)	1,71 (1,11–2,63)	0.016
Genetic model[b]					
Codominant	G/G	71 (61.2)	45 (44.1)	1 (ref.)	
	G/A	41 (35.3)	50 (49)	1.81 (1.03–3.18)	0.081
	A/A	4 (3.5)	7 (6.9)	2.29 (0.62–8.46)	
Dominant	G/G	71 (61.2)	45 (44.1)	1 (ref.)	
	G/A & A/A	45 (38.8)	57 (55.9)	1.85 (1.07–3.21)	0.027
Recessive	G/G & G/A	112 (96.5)	95 (93.1)	1 (ref.)	
	A/A	4 (3.5)	7 (6.9)	1.74 (0.48–6.24)	0.39
Additive	–	–	–	1.69 (1.05–2.70)	0.028

[a] Univariate analysis by Fisher's exact test.
[b] Logistic regression adjusted by sex and age.

Table 2
Distribution of *PTEN-L* genotypes according to gender.

Gender	Genotype	Controls (%)	Patients (%)	OR (95% CI)	p
F	GG	34 (56.7)	26 (47.3)	1 (ref)	
	GA/AA	26 (43.3)	29 (52.7)	1.46 (0.69–3.04)	0.355
M	GG	37 (66.1)	20 (41.3)	1 (ref)	
	GA/AA	19 (33.9)	28 (58.7)	2.77 (1.23–6.20)	0.0165[a]

Fisher's exact test.
[a] Statistically significant $p < 0.05$.

Table 3
Distribution of *PTEN-L* genotypes according to age.

Age	Genotype	Controls (%)	Patients (%)	OR (95% CI)	p
< 45	GG	31 (62)	18 (48.65)	1 (ref)	
	GA/AA	19 (38)	19 (51.35)	1.26 (0.86–1.85)	0.275
> 45	GG	40 (60.61)	27 (41.54)	1 (ref)	
	GA/AA	26 (39.39)	38 (58.46)	2.165 (1.07–4.35)	0.0363[a]

Fisher's exact test.
[a] Statistically significant $p < 0.05$.

Table 4
Main clinical parameters stratified according *PTNE-Long* genotypes.

Clinical parameters	Genotypes		p value
	G/G n (%)	G/A & A/A n (%)	
CML phase			
Chronic (n = 96)	43 (45)	53 (55)	0.69
Accelerated (n = 6)	2 (33)	4 (67)	
Treatment outcome			
TKIs responders (n = 43)	18 (42)	25 (58)	0.687
TKIs non-responders (n = 59)	28 (47)	31 (53)	
BCR-ABL1 mutation non-responders			
Yes (n = 16)	7 (44)	9 (56)	0.135
No (n = 43)	29 (67)	14 (33)	
Molecular response			
Major/4.5/5.0 (n = 43)	18 (42)	25 (58)	0.687
Minor/minimal/null (n = 59)	28 (47)	31 (53)	
Cytogenetic response			
CCR (n = 52)	21 (40)	31 (60)	0.550
Minimal/minor/null (n = 50)	24 (48)	26 (52)	
Treatment			
ITK 1st generation (n = 40)	19 (47)	21 (53)	0.841
ITK 2nd–3rd generation (n = 62)	28 (45)	34 (55)	

Fisher's exact test. CCR: complete cytogenetic response.

p = 0.0165. No statistical differences were found in the younger group of patients and among women.

The association between *PTEN-L* rs12573787 and the main clinical-pathological characteristics of CML patients was analyzed by grouping genotypes according the dominant model (G/G vs G/A + A/A) (Table 4). The analysis of *ABL1* kinase domain mutation was performed in 59 patients with TKI resistance indicating that 16 patients show 18 mutations, seven of them affecting ATP-binding site, six on the P-loop and five on the catalytic domain of ABL1. The most common mutations were T315I, V299L, G250E and F359V. No significant differences were observed between any of the clinical parameters and the genotype distribution.

The analysis of overall survival stratified by SNP rs12573787 genotypes considering the dominant model showed no significant differences between groups (data not shown). Likewise, no significant differences were found in overall survival among CML patients (p = 0.483) and resistant patients (p = 0.481).

To compare the frequencies of *PTEN-L* alleles G and A from our control group with those from Caucasian, Asian and African populations, we used data reported in the 1000 Genomes Project (http://browser.1000genomes.org), which provides a deep characterization of human genome sequence variation. This comparison showed that allele frequencies from our cohort are similar to the general frequency reported from admixed American populations including those from Colombia, Mexico, Peru and Puerto Rico sub-populations. The frequency of the A allele from the Argentine general population was lower than those observed in the East Asian general population and higher than African, European and South Asian populations (Fig. 2).

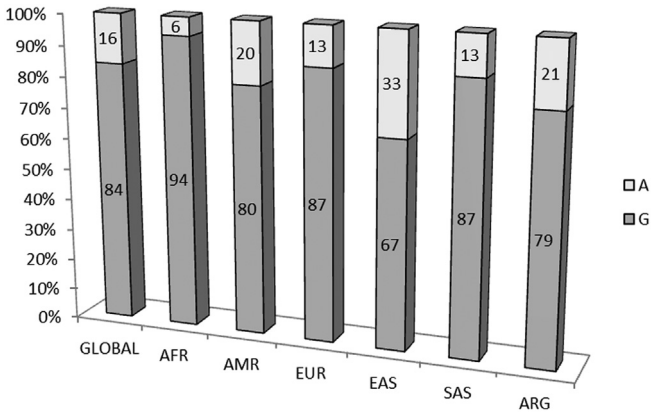

Fig. 2. Distribution of *PTEN-L* rs12573787 SNP worldwide. AFR: African, AMR: Ad Mixed American, ARG: Argentine, EAS: East Asia, EUR: European, SAS: South Asian.

4. Discussion

In this case-control study, we assessed the association of genetic variants in *PTEN-L* gene with CML susceptibility and clinical outcome in Argentine population. The A-allele of rs12573787 SNP (NM_000314.4:c.-510G > A) was found to be associated with high CML risk. In contrast, no association was observed between *PTEN-L* rs12573787 SNP genotypes and the response to TKIs treatment.

Accumulated evidence demonstrated that germline polymorphisms can determine cancer susceptibility and/or the response to a given therapy (Ko et al., 2016). PTEN is a phosphatase enzyme that is frequently altered in cancer. Mutations on *PTEN* gene can lead to deregulation of protein synthesis, cell cycle, DNA repair, cell migration, growth and cell survival signaling (Hopkins et al., 2014). Even though PTEN involves lipid phosphatase-independent functions in other cellular contexts, PTEN loss-induced lymphomagenesis and leukemogenesis have been mostly linked to the activation of the PI3K-AKT pathway (Gutierrez et al., 2009; Tesio et al., 2016; Malek et al., 2017). PTEN has been reported to play an important role in modulating proliferation and apoptosis by decreasing the levels of PIP3 that determine AKT activation. In vitro overexpression of *PTEN-L* inhibited migration of cells and this function was dependent on its lipid phosphatase activity (Wang et al., 2015). It has been described that loss of PTEN function is consequence of point mutations, genomic deletions and micro-deletions (Hieronymus et al., 2017). PTEN-L is the product of the alternative translation initiation, which represents a mechanism of producing multiple proteins from a single transcript (Malaney et al., 2013).

Since *PTEN-L* is evolutionarily conserved (Hopkins et al., 2013), mutations or polymorphic variants may play a significant role in PTEN-L function. Our previous studies of canonical *PTEN* in CML patients indicate no direct association between gene expression and treatment resistance (Ferri et al., 2014). However, low PTEN protein expression in mouse models with CML and cell culture, have been observed (Peng et al., 2010; Huang et al., 2014). Canonical *PTEN* expression could not be reflected in the studies carried out, since the same mRNA encodes two different proteins, and protein studies should be done to draw major conclusions. Since the role of PTEN-L in leukemogenesis is remarkable (Malaney et al., 2013; Park et al., 2010), functional empirical studies are necessary to elucidate its involvement in CLM.

Few studies have addressed *PTEN* polymorphisms. It was suggested that the SNP rs12573787 is unlike to influence on the susceptibility to hereditary and non-hereditary prostate cancer (Xie et al., 2011). Similar results were observed for endometrial cancer (Wang et al., 2012). It should be noted that these both studies corresponds to *PTEN* gene nomenclature and the analysis was previous to the discovery of PTEN-L protein. Considering the relative location of the *PTEN-L* initiation

codon, CUG, and the SNP rs12573787 only 10 nucleotides downstream from the initiation codon of *PTEN-L*, c.10G > A change may disturb the 3D-structure of *PTEN-L* mRNA at this point impacting PTEN-L translation initiation and consequently its expression. Despite these functional speculations, we demonstrated a neat association between *PTEN-L* rs12573787 polymorphism and CML development when compared to the control group. As genetic and non-genetic factors, such as individual gender and age, may influence the susceptibility to develop CML, we evaluated the exposure to *PTEN-L* rs12573787 genotypes within these strata (i.e., male vs females, and < 45 vs > 45 years old). As it was reported, CML is more frequent in males (Berger et al., 2005; Höglund et al., 2015) and, in line with this evidence; we found that the genetic risk of the *PTEN*-L rs12573787 for developing CML is confined to this gender group. Unexpectedly, a statistical association between *PTEN-L* rs12573787 genotypes and CML was only found in patients older than 45 years old.

5. Conclusion

To our knowledge, this is the first time that *PTEN-L* rs1257378 was studied in CML. This study indicates that the allele A of *PTEN-L* rs1257378 is a risk factor for development of CML in our Argentine population. In contrast, no association was found with the response to CML treatment with TKIs.

Conflicts of interest

Authors declare no conflict of interests.

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