Methylation of CpG sites in *BCL2* major breakpoint region and the increase of *BCL2/JH* translocation with aging

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Abstract The *BCL2* breakage mechanism has been shown to be highly dependent on DNA methylation at the major breakpoint region (MBR) CpG sites. We recently described an increased frequency of *BCL2/JH* translocation with aging. It is known that methylation levels change with aging. The present study aimed to determine whether methylation alterations at CpG sites of *BCL2* MBR were the cause of increased breakages with aging. We analyzed the methylation levels of

three CpG sites on the region by pyrosequencing and studied if methylation levels and/or polymorphisms affecting CpG sites were associated with an increase of translocations. We observed that although the methylation levels of MBR CpG sites were higher in individuals with *BCL2/JH* translocation, in contrast to our expectations, these levels decreased with the age. Moreover, we show that polymorphisms at those CpG sites leading to absence of methylation seem to be a protective factor for the apparition of translocations.

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Introduction

Many of the causes of spontaneous chromosomal translocations in humans are still unexplained. Nevertheless, double-strand DNA breaks (DSBs) are known to be required in all proposed mechanisms (Raghavan et al. 2004). The most common translocation in human cancer is $t(14;18)(q32;q21)$, which is a characteristic of follicular lymphomas (FL), although it has been also detected in 40–60 % of healthy population (Nambiar and Raghavan 2012). This translocation juxtaposes the *BCL2* locus on chromosome 18 to the enhancer element of the immunoglobulin heavy-chain (IgH) locus on chromosome 14 in pre-B cells (Bakhshi et al. 1985, 1987), leading to *BCL2* overexpression.

The break on chromosome 14 is known to be induced by erroneous V(D)J recombination mechanism

(Nambiar and Raghavan 2013), responsible in normal conditions for the generation of antigen receptor diversity in higher eukaryotes (Lewis 1994; Schatz and Baltimore 2004). However, the mechanism of breakage at the *BCL2* gene has been less studied. Remarkably, although the *BCL2* gene is more than 200 kb long, most of the breaks occur within a small 150-bp region located in the 3'-untranslated region (3'-UTR) of the third exon, known as the major breakpoint region (MBR) (Cleary and Sklar 1985; Jäger et al. 2000).

Recent studies point out that *BCL2* MBR adopts a non-B DNA structure in genomic DNA (G-quadruplex), which contributes to chromosomal fragility due to the single-stranded character at the region (Nambiar et al. 2011; Zhao et al. 2010). Moreover, the analysis of breakpoint junctions in lymphoma patients displayed that MBR breaks focused on three CpG peaks (Tsai et al. 2008), suggesting that CpG sites are hotspot for breaks in MBR. Nowadays, it is known that this breakage process is highly dependent on the activation-induced cytidine deaminase (AID) enzyme, which acts at the methylated forms of these CpG sites to deaminate the methyl C to yield a T. The resulting TG mismatch is converted to DSBs with the contribution of the RAG complex and Artemis (Cui et al. 2013). This implies that individuals whose pro-B/pre-B cells are methylated at the CpGs within MBR would be susceptible to rearrangements (Tsai et al. 2008).

Recently, we described a higher frequency of *BCL2/JH* rearrangements with aging (Martín-Guerrero et al. 2014). It is widely accepted currently that aging is associated with profound epigenetic changes (Heyn et al. 2012). Therefore, we can hypothesize that the increased frequency of breakages with aging could be due to methylation changes at CpG sites within *BCL2* MBR. Additionally, we should also consider that polymorphisms at those CpG sites could alter the methylation levels of those CpG sites and in turn influences on the breakage.

Consequently, the present study aimed to determine whether methylation alterations at CpG sites of *BCL2* MBR (hotspot for breaks) are the cause of increased breakages with aging. We analyzed the methylation levels of three CpG sites on the region and studied if methylation levels and/or polymorphisms were associated with an increase of translocations.

Material and methods

Study population

A total of 565 blood samples from Spanish healthy individuals and 33 follicular lymphomas with available formalin-fixed paraffin-embedded tissue were included in the study. Healthy individuals were collected at the Araba University Hospital, University of the Basque Country (UPV/EHU), University Hospital Cruces, and Hospital General Valencia from 2004 to 2007 and are deposited in the C.001171 collection of the Carlos III Health Institute (Table 1). Information about age and sex were recorded for each individual. Follicular lymphoma cases were collected at University Hospital Donostia. The study was approved by the Ethical Committees of all institutions. Informed consent was obtained from all patients, or from their parents, before sample collection, following the Spanish Organic Law 15/1999. Genomic DNA was extracted from peripheral blood using conventional procedures.

Study of *BCL2* breakpoints

BCL2/JH translocation involving the major breakpoint region (MBR) of the *BCL2* gene was analyzed in 545 healthy individuals and 33 follicular lymphomas (FL) as previously described (Martín-Guerrero et al. 2014), obtaining a total of 206 and 14 positive samples, respectively. To identify the DNA breakpoints, PCR products from 45 positive healthy individuals and 14 follicular samples were evaluated by direct sequencing, using an ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems) at the General Research Services (SGIKER) of the University of the Basque Country. Sequences were analyzed using Chromas 2.33. Analyses in silico through the TICdb v.3.0 (<http://www.unav.es/genetica/TICdb>) and literature search were also performed to obtain all the MBR breakpoints described until now.

Polymorphisms genotyping

The polymorphisms rs4987855 and rs4987856 were genotyped by PCR-restriction fragment length polymorphism (RFLP) with BstUI and High Resolution Melting (HRM) technologies, respectively, in 565 healthy individuals. Primers sequences and PCR conditions are described in Supplemental Table 1. To confirm the

Table 1 Characteristics of the healthy study population

Total number of participants	565	
Age (mean, sd)	37.21±18.18	
Age range (years)	1–84	
Gender	<i>n</i>	%
Male	321	56.8
Female	244	43.2
Study populations	<i>n</i>	Age range
<i>BCL2-JH</i> detection	545	1–84
<i>BCL2</i> breakpoint characterization	45	1–84
SNPs at MBR CpG sites genotyping	565	1–84
CpG methylation	93	<20 years old (<i>n</i> =40) >40 years old (<i>n</i> =53)

specificity of the PCR products, the different genotypes were analyzed by direct sequencing as previously described.

Pyrosequencing

The methylation status of specific MBR CpG sites was determined using pyrosequencing technology in 40 individuals <20 years old and 53 >40 years old. Genomic DNA was bisulfite converted using the EpiTect Bisulfite Conversion Kit (Qiagen), according to manufacturer's instructions. The pyrosequencing assay was designed using PSQ Assay Design software (Qiagen). Bisulfite pyrosequencing was performed on PyroMark Q24 system according to standard protocols and evaluated with the analysis software Pyro Q-CpG (Qiagen), which was also used to quantify the percentage of methylated cytosines at the analyzed CpG sites. Methylation percentage values lower than 10 % were not considered. All assays were optimized and validated using commercially completely methylated DNA (Millipore) as the positive control and DNA amplified with illustra GenomiPhi V2 kit (GE Healthcare) as the negative control. Water blanks and genomic DNAs were included with each assay. PCR and primer sequences are shown in Supplemental Table 2.

Statistical analyses

The frequency of *BCL2/JH* translocation was defined as the proportion of individuals positive for the translocation with respect to the total population. Statistical CpG

methylation differences between groups were examined with *T* test using PASW Statistics software version 23 (SPSS Inc., Chicago, IL). Statistical significance was set at $P<0.05$.

Results

Study of *BCL2* breakpoints

A total of 36 different breakpoints were detected. Sequence analysis of *BCL2/JH* breakpoints from 45 positive healthy individuals displayed a total of 24 different breakpoints. The sequencing of 14 follicular lymphomas added 7 breakpoints, from which 2 were coincident with those found in healthy people. Additionally, 11 *BCL2* breakpoints were found to be described in the TICdb v.3.0 and literature, 4 of which were coincident with breakpoints from healthy people ($n=2$) and follicular lymphomas ($n=2$). All breakpoints were located in a region of 201 bp of the MBR region. Remarkably, 32/70 (45.7 %) breakpoints were next to one of the six CpG sites present in the MBR region (average mean 3.89 bp) (Fig. 1).

Methylation of the CpG sites at *BCL2* MBR region

Three out of six CpG sites present in the *BCL2* MBR region were selected for methylation analyses, due to their proximity to the breakpoints identified in healthy individuals and to the G-quadruplex region. However, since two of them were polymorphic (rs4987855, A/G;

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GACCTTTAGAGAGTTGCTTTACCGTGGCCTGTTTCAACACAGACCCACCCAGAGCC*
1CTCCTGCC*1CTCC*1T*1T*1C*1&1CG*1CGG*1GGGCTTT*1CTCA2TGGCTG*1TCCTT
CAGGGTC*1T*1TCCTGAAAT*1GCAG*1TG*2G*1TG*1C*1T*2T*1A*1#1C*8G*9&7C*1TCC
ACCAAGA*1&1AAGCAGGAAA*1CCTGTGGTATGAAGCCA*1G*4ACC*1TCC*2C*1CGGC
#1&1GG*1GCCTCAGGGAACAGAATGATCAGACCTTTGAATGATTCTAATTTTTAAGCA
AAATATTATTTTATGAAAGGTTT*1A*1CATTGTCAAAGTGATGAATATGGAATATCCAAT

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Fig. 1 Breakpoints and CpG sites detected within the *BCL2* MBR region (5'-3'). The symbol *asterisk* corresponds to breakpoints found in 45 healthy people positive for *BCL2/JH* translocation. The symbol *ampersand* corresponds to breakpoints detected in 14 FL patients. The symbol *number sign* corresponds to 11

breakpoints described in TICdb v.3.0 and literature. The *super index* indicates the number of individuals with that breakpoint. The CpG sites appear in **bold** and the G-quadruplex sequence underlined

rs4987856, A/G) (Fig. 1), we first analyzed whether the methylation status of these two CpG sites was modified depending on SNP genotypes. For that, we first genotyped a total of 565 healthy individuals, obtaining a success rate of 93.3 % for rs4987855 and 91.5 % for rs4987856. The genotype frequencies were described in Supplemental Table 3. We next quantified the methylation status of the two polymorphic CpG sites in 93 samples, observing for both SNPs significant differences in the methylation levels between genotypes: individuals homozygous for rs498755 G allele ($n=46$) showed a significantly higher methylation level than heterozygous ($n=40$) (27.74 vs 18.64 %; $P<0.001$). Similar results were obtained when homozygous for rs498756 G allele ($n=49$) were compared to heterozygous ($n=29$) (40.72 vs 26.55 %; $P<0.001$) (Fig. 2a). For both SNPs, individuals homozygous for A allele showed absence of methylation (data not shown).

Given that the genotype affected the methylation levels, to avoid false associations because of the genotype, we selected a study population of 93 individuals matched by genotype (40<20 and 53>40 years old) and analyzed the methylation levels of the three CpG sites previously selected at *BCL2* MBR region. We obtained average methylation levels of 50.52 % at CpG site 1, 23.51 % at CpG site 2, and 35.26 % at CpG site 3.

Methylation of the CpG sites at *BCL2* MBR region and age

Considering the age, individuals <20 years old showed higher methylation levels in comparison with people >40 years old (CpG site 1, 55.23 vs 46.95 %, $P=0.008$; CpG site 2, 26.34 vs 21.37 %, $P=0.01$; CpG site 3, 41.41 vs 30.41 %, $P<0.001$) (Fig. 2b).

CpGs methylation and *BCL2/JH* translocation

To determine if the methylation was related with the risk of occurring translocations, we analyzed the association between the methylation levels of these three CpG sites and the presence of *BCL2/JH* translocation. Surprisingly, individuals with the translocation showed in general higher methylation values, showing CpG sites 1 and 2 significant results (CpG site 1, 54.46 vs 45.98 %, $P=0.006$; CpG site 2, 25.71 vs 20.97 %, $P=0.01$) (Fig. 2c).

rs4987855 and rs4987856 genotypes and *BCL2* MBR breakage

To determine the involvement of rs4987855 and rs4987856 A/G alleles on *BCL2/JH* translocation, we selected individuals positive for the translocation heterozygous for the polymorphisms and analyzed which allele was present at the translocated strand by sequencing. If no allele was involved in the translocation, the breaks should happen equally. However, when we sequenced the translocated strand from 37 heterozygote individuals (7 follicular lymphomas and 30 healthy samples), we observed that all follicular samples (7/7) had the G allele at the translocated strand. Among the healthy individuals, 35/37 showed the G allele at the translocated strand (Fig. 3).

Discussion

Previous studies of the *BCL2* breakpoint regions detected that most breaks occur in three clusters in proximity to CpG sites in the MBR of *BCL2* (Tsai et al. 2008; Tsai and Lieber 2010). However, the majority of these

Fig. 2 Methylation levels for (a) rs4987855 and rs4987856 based on genotypes, (b) three CpG sites within the *BCL2* MBR according to the age group, and (c) three CpG sites within the *BCL2* MBR depending on the presence or absence of translocation

studies were performed in patients with FL or cell lines and little has been done in relation with the age in healthy people. The present study showed that from 45 breakpoints analyzed in healthy people, 46.7 % (21/45) were next to one of the six CpG sites present in the MBR

region, with an average distance to the nearest CpG site of 3.89 bp. Similar data were displayed by Tsai et al., who observed that 43 % of breakpoints (208/487) occurred near CpG sites with an average distance of 4.4 bp, compared to 11.2 bp if the breakpoints had been

Fig. 3 *BCL2/JH* fusion gene sequence in a healthy individual heterozygous for rs4987855 and rs4987856. The black bar indicates the breakpoint

randomly distributed (Tsai et al. 2008). These findings confirm previous studies indicating that CpG sites are hotspots for DSBs in the *BCL2* MBR (Cui et al. 2013).

Moreover, the methylation of these CpG sites was suggested to be an important event for directing breakage (Cui et al. 2013). To determine if CpG methylation was decisive for the occurrence of translocations in *BCL2* MBR, we analyzed the methylation levels of three CpG sites close to the most recurring breakpoints found in the healthy population and to the G-quadruplex region. Since two of these were polymorphic, we firstly checked the correlation genotype-methylation, finding that effectively the loss of CpG sites due to the genotype affected the methylation levels. Consequently, to avoid false associations because of the genotype, we studied the methylation levels of the three CpG sites considering the genotype. We observed that all CpG sites analyzed were methylated in almost all individuals with the translocation, suggesting that this mechanism was important for DNA breakage. Supporting this idea, Cui et al. showed that CpG sites must be methylated to be susceptible to a breakage process initiated by activation-induced deaminase (AID) enzyme, RAG and Artemis to create DNA lesions (Cui et al. 2013).

Additionally, we observed that although methylation levels of MBR CpG sites were higher in individuals with *BCL2/JH* translocation (39.24 vs 33.23 %, average methylation), these levels decreased with the age (40.99 % <40 years old vs 32.91 % >40 years old, average methylation). This means that the increase of translocations with aging previously described by our group (Martín-Guerrero et al. 2014) cannot be explained by a CpGs methylation increase. Moreover, although there was no association with aging, our study showed that higher methylation levels led to a higher frequency of *BCL2/JH* translocations. One possible explanation could be that higher methylation levels make AID enzyme to deaminate the methyl C to yield a T with a higher frequency, increasing the number of mutations T:G and in turn increasing the probability of translocations. Therefore, the lack of methylation at MBR CpG sites because of polymorphisms or demethylation would decrease the risk of translocations.

Finally, to check if CpGs methylation must be a prerequisite for the breakage, we analyzed the translocated strands in heterozygote individuals for one of the two polymorphisms of those CpG sites (rs4987855 and rs4987856). The 94.6 % of heterozygote individuals showed the G allele in the translocated

strand, indicating that G allele is important for the translocation to occur. According with these results, CpG methylation appears to be an imperative requirement for translocations. Interestingly, a recent study in human pre-B and primary pre-B cell lines demonstrated that average methylation frequency in the *BCL2* MBR is necessary for occurring translocations and was significantly lower than in the adjacent DNA (62.5 vs 92.5 %) (Lu et al. 2015).

In summary, we show that the breakage mechanism at *BCL2* MBR is highly dependent on the CpG sites methylation but this is not related with aging. The genotype that eliminates CpGs could act as a protective mechanism to the apparition of *BCL2/JH* translocation.

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Conflict of interest The authors declare that they have no competing interests.

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