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Methylation of the nonhomologous end joining repair pathway genes does not explain the increase of translocations with aging

Idoia Martín-Guerrero · Elena de Prado · Elixabet Lopez-Lopez · Maite Ardanaz · Juan Carlos Vitoria · Luis A. Parada · Cristina García-Orad · Africa García-Orad

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Abstract Chromosome translocations are especially frequent in human lymphomas and leukemias but are insufficient to drive carcinogenesis. Indeed, several of the so-called tumor specific translocations have been detected in peripheral blood of healthy individuals, finding a higher frequency of some of them with aging. The inappropriate repair of DNA double strand breaks by the nonhomologous end joining (NHEJ) pathway is one of the reasons for a translocation to occur. Moreover, fidelity of this pathway has been shown to decline with age. Although the mechanism underlying this inefficacy is unknown, other repair pathways are inactivated by methylation with aging. In this study, we analyzed the implication of NHEJ genes methylation in the increase of translocations with the age. To this aim, we determined the relationship between translocations and aging in 565 Spanish healthy individuals and correlated these data with the methylation status of 11 NHEJ genes. We found higher frequency of *BCL2-JH* and *BCR-ABL* (*major*) translocations with aging. In addition, we detected that two NHEJ genes (*LIG4* and *XRCC6*) presented age-dependent promoter methylation changes. However, we did not observe a correlation between the increase of translocations and methylation, indicating that other molecular mechanisms are involved in the loss of NHEJ fidelity with aging.

Keywords DNA methylation · Chromosome translocation · Healthy individual · NHEJ · Age

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I. Martín-Guerrero · E. de Prado · E. Lopez-Lopez · A. García-Orad (✉)
Department of Genetics, Physical Anthropology and Animal Physiology, Faculty of Medicine and Odontology, University of the Basque Country UPV/EHU, Barrio Sarriena sn, 48940 Leioa, Bizkaia, Spain
e-mail: africa.garciaorad@ehu.es

A. García-Orad
BioCruces Health Research Institute, Barakaldo, Bizkaia, Spain

M. Ardanaz
Hospital Txagorritxu, Vitoria, Spain

J. C. Vitoria
Hospital de Cruces, Barakaldo, Spain

L. A. Parada
Institute of Experimental Pathology, UNSa-CONICET, Salta, Argentina

C. García-Orad
Assistance to primary health care center -Torrent 1, Hospital General Valencia, Valencia, Spain

Introduction

Chromosomal translocations are characteristic hallmarks of many cancers, especially lymphoma and leukemia (Nambiar and Raghavan 2013), resulting in the deregulated expression of oncogenes or the generation of novel fusion genes. However, despite being necessary, translocations are insufficient to drive carcinogenesis and additional genetic changes are required to achieve

full transformation (Janz et al. 2003). Indeed, several translocations have been observed in lymphocytes from healthy individuals, including *BCL2-JH*, *BCR-ABL*, *NPM-ALK*, *BCL1-JH*, or those involving *BCL6* and *MLL* genes (Biernaux et al. 1995; Bose et al. 1998; Brassesco et al. 2009; Bäsecke et al. 2002; Janz et al. 2003; Maes et al. 2001; Rabkin et al. 2008; Schüler et al. 2003). Notably, diverse studies have shown that the frequency of these translocations increase with aging (Dölken et al. 2008; Schüler et al. 2003). Although the mere presence of these translocations is not sufficient to trigger cancer development, a recent study has related the presence of *BCL2-IGH* translocation in blood from healthy individuals with the higher risk of developing follicular lymphoma (FL) (Roulland et al. 2014).

For a translocation to occur, one of the criteria that needs to be fulfilled is the inappropriate repair of DNA double strand breaks (DSBs) (Mani and Chinnaiyan 2010). In mammalian cells, this repair is preferentially performed by the nonhomologous end joining (NHEJ) pathway (Lieber 2010). Diverse studies have suggested that fidelity of NHEJ repair declines with aging (Gorbunova and Seluanov 2005; Karanjawala and Lieber 2004; Seluanov et al. 2004), which may lead to the increased genomic instability in the elderly. Although the molecular mechanism underlying this inefficacy of the NHEJ pathway with aging is still unknown, it has been demonstrated that other repair pathways are inactivated by the methylation of their genes (Thompson et al. 2010). Specifically, several DNA repair and protection genes, such as *hMLH1*, *MGMT*, or *GSTP1*, have been shown to be methylated with aging (Christensen et al. 2009; Fraga and Esteller 2007). Therefore, it can be expected that NHEJ genes may also gain methylation with aging and this could lead to an increase in translocations.

To check this hypothesis, we determined the relationship between four tumor-specific chromosome translocations present in healthy individuals (*BCL2-JH*, *BCR-ABL*, *MLL-AF4*, and *TEL-AML1*) and aging. Next, we correlated the presence of these translocations with the methylation status of 11 NHEJ genes.

Material and methods

Study population

A total of 565 Spanish healthy individuals were included in the study (Table 1). Blood samples were collected

Table 1 Characteristics of the study population

Total number of participants	565	
Age (mean, SD)	37.21±18.18	
Age range (years)	1–84	
Gender	Number	Percent
Male	321	56.8
Female	244	43.2
Study populations	Number	Age range
BCL2-JH	545	1–84
BCR-ABL, *MLL-AF4*, *TEL-AML1*	171	1–84
NHEJ pathway methylation	182	<20 years old (*n*=91) >40 years old (*n*=91)

at the Araba University Hospital, University of the Basque Country, University Hospital Cruces and General Valencia Hospital from 2004 to 2007 and are deposited in the C.001171 collection of the Carlos III Health Institute. Information about age and sex were recorded for each individual. 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 and RNA were extracted from peripheral blood using conventional procedures.

Detection of the *BCL2-JH* translocation

BCL2-JH translocation involving the major breakpoint region (MBR) of the *BCL2* gene was analyzed in 1 μg of DNA, using a nested polymerase chain reaction (n-PCR). Twenty samples were eliminated because there was not enough material for the analysis (<1 μg DNA). Of the total of 565 individuals, 545 were finally evaluated. Primers sequences and PCR conditions are described in Supplemental Table 1. The sensitivity to detect the translocation, determined by a serial dilution of the Karpas 422 cell line, was one in 10^6 normal cells.

Detection of the *BCR-ABL*, *MLL-AF4*, and *TEL-AML1* translocations

To investigate the occurrence of the translocations *BCR-ABL* (involving major and minor regions), *MLL-AF4*, and *TEL-AML1*, a total of 500 ng of cDNA from 171 individuals with available RNA was analyzed in a single

assay by a multiplex n-PCR using a commercial kit (Qiagen, Hilden, Germany). The first round was performed with external primers, and the second one with reverse internal primers marked with fluorescent dye at their 5′-end following a previously published protocol (Marín et al. 2001). The sequences of primers used in both rounds of amplifications are described in Supplemental Table 2. The cell lines SD1, Mv4-11, and REH, harboring the *BCR-ABL*, *MLL-AF4*, and *TEL-AML1* translocations, respectively, were used as positive controls. In addition, negative controls were included in all reactions. All amplified products were subsequently characterized by Genescan analysis in the ABI 3100 genetic analyzer at SGIker. Samples displaying a pick at the expected size by capillary electrophoresis were considered positive.

Methylation-specific PCR

From the total of 565 individuals, 91≥40 and 91≤ 20 years old were selected for methylation analyses (Table 1). *RAG1*, *RAG2*, *ATM*, *ATR*, *XRCC4*, *XRCC5*, *NHEJ1*, *DCLRE1C*, *PRKDC*, *XRCC6*, and *LIG4* genes, all belonging to the NHEJ DNA repair pathway, were selected for methylation-specific PCR (MSP). We used the UCSC Genome browser (genome.ucsc.edu/) to identify CpG islands within these genes, and their methylation was analyzed by MSP. Briefly, genomic DNA was converted by sodium bisulfite treatment using the CpGenome™ Fast DNA Modification Kit (Chemicon® Int), according to the manufacturer's instructions. The quality of the converted DNA was verified with the amplification of the *ACTB* gene. Primer sequences and PCR conditions are described in Supplemental Table 3. DNA amplified with illustra GenomiPhi V2 kit (GE Healthcare) was used as the negative control and CpGenome universal methylated human DNA (Millipore, Schwalbach, Germany) was the positive control. Water blanks and genomic DNAs were included with each assay.

Statistical analyses

The frequency of each translocation was defined as the proportion of individuals positive for the translocation with respect to the total population. A linear regression model was used to analyze the relationship between the age and frequency of translocations. The differences between groups were analyzed by the chi-square test using the STATA 8.0 software.

Promoter methylation was given as the percentage of the methylated-positive individuals. Statistical differences between groups were examined with the chi-square test using PASW Statistics software version 18 (SPSS Inc., Chicago, IL). Statistical significance was set at $P<0.05$.

Results

Frequency of the *Bcl2-JH* translocation

The t(14;18)-MBR translocation was found in 206 (37.8 %) of the 545 individuals evaluated. We studied the effect of aging, stratifying the population in eight consecutive groups. Analysis of data showed a gradual increase from 21.4 % in the group of children (1–9 years old) to 60 % among people older than 70 years (Table 2). Comparison of the translocation frequencies in each group with that found in the youngest group demonstrated that the frequency of the t(14;18) started to be significantly different at the age of 40. To verify this tendency, we divided our population in individuals younger (n=298), and older (n=247) than 40 years and compared the frequency of translocation in these two groups. We observed a significant higher frequency in adults (46.2 %, 114/247) than in the group of young people (30.9 %, 92/298) (P=0.0002) (Fig. 1).

Frequency of the *BCR-ABL*, *MLL-AF4*, and *TEL-AML1* translocations

Out of the 171 healthy individuals analyzed, the translocation *BCR-ABL* major was found in 17 (9.9 %), the *BCR-ABL* minor in 2 (1.2 %), and the *MLL-AF4* in 11 individuals (6.4 %). TEL-AML1 rearrangement was not detected in any individual.

The frequency of the *BCR-ABL major* and *MLL-AF4* translocations were found to be related to aging: the frequency of *BCR-ABL* major translocation increased significantly with the age of the individuals from 4.8 % (4/83) in the group of people younger than 40 years old to 14.7 % (13/88) among older individuals (P=0.004). In contrast, MLL-AF4 translocation was more rare among individuals older than 40 years old (1/88; 1 %) than in the group under this age (10/83; 12 %) (P=0.004) (Fig. 1).

Table 2 Prevalence of t(14;18) by age groups

Range age (years)	Number	t(14;18), number (%)	OR	*P* value	Confidence interval (95 %)
1–9	42	9 (21.4)	1.00	–	–
10–19	79	28 (35.4)	2.01	0.115	0.84–4.80
20–29	78	18 (23.1)	1.10	0.837	0.44–2.72
30–39	99	37 (34.4)	2.18	0.068	0.94–5.07
40–49	107	48 (44.9)	2.98	0.010	1.30–6.83
50–59	81	34 (42.0)	2.65	0.026	1.12–6.26
60–69	39	20 (51.2)	4.34	0.001	1.77–10.66
>70	20	12 (60.0)	4.62	0.001	1.84–12.55

OR odds ratio

Methylation status of NHEJ genes

To determine the existence of association between the presence of translocations and the inefficacy of the NHEJ pathway because of epigenetic mechanisms, we analyzed the methylation status of 11 genes encoding proteins involved in the NHEJ pathway.

Our in silico analysis showed that the *RAG1* and *RAG2* genes do not contain CpG islands, and therefore were excluded from the study. Among the remaining genes, seven (*ATM*, *ATR*, *XRCC4*, *XRCC5*, *NHEJ1*, *DCLRE1C*, and *PRKDC*) displayed one CpG island each and the other two genes (*XRCC6* and *LIG4*) presented two each. In total, 11 CpG regions in the promoter of nine genes were analyzed in 182 healthy individuals (91≥40 and 91≤20 years old).

We found that the frequency of samples showing methylation was in general low (<5 %), except for the *XRCC5* gene promoter which was methylated in 50 % of the samples (Table 3). *ATR*, *XRCC6* region 1, and *LIG4* region 1 showed methylation in 4, 5, and 3 % of the samples, respectively. *XRCC4*, *DCLRE1C*, *NHEJ1*, *ATM*, and *LIG4* region 2 showed methylation percentages ranging from 0.5 to 2 %. *PRKDC* and *XRCC6*

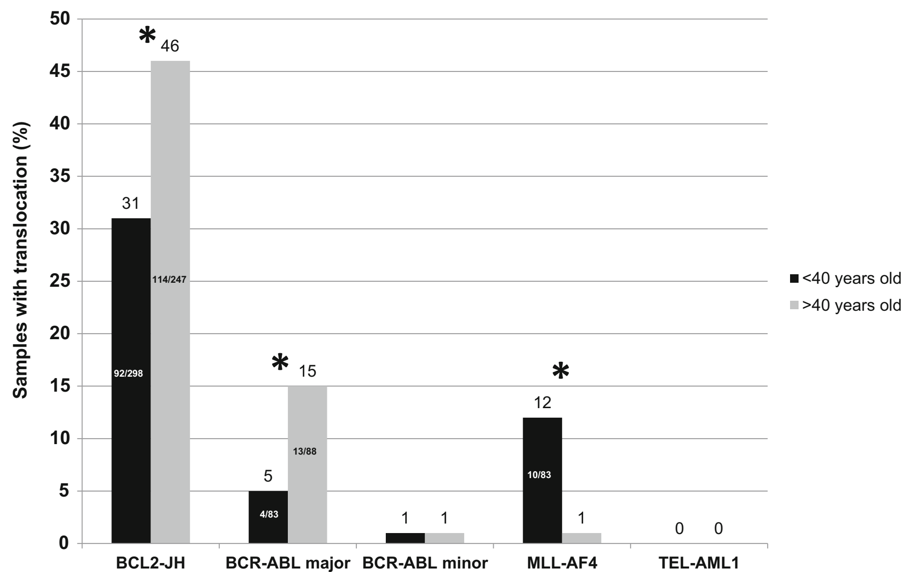

Fig. 1 Prevalence of translocation positivity according to the age of individuals. For *BCL-JH*, a total of 545 individuals were analyzed (298<40 vs 247>40 years old). For the *BCR-ABL*, *MLL-AF4* and *TEL-AML1* chromosome translocations, 171 individuals were studied (83<40 vs 88>40 years old). *Asterisk* represents statistically significant results

Table 3 Methylation status of nine NHEJ genes in 182 healthy individuals

CpG island	MSP + Number (%)	MSP − Number (%)
ATR	8 (4)	174 (96)
XRCC4	1 (0.5)	181 (99.5)
XRCC5	91 (50)	91 (50)
XRCC6 CpG1	10 (5)	172 (95)
XRCC6 CpG2	0 (0)	182 (100)
DCLRE1C	1 (0.5)	181 (99.5)
NHEJ1	5 (3)	177 (97)
LIG4 CpG1	5 (3)	177 (97)
LIG4 CpG 2	3 (1.6)	179 (98.4)
ATM	2 (1)	180 (99)
PRKDC	0 (0)	182 (100)

MSP methylation-specific PCR, + positive, − negative

region 2 showed no methylation. Remarkably, 12.1 % of all individuals showed methylation in more than one CpG island.

Considering the age of individuals, individuals >40 years old showed in general a higher methylation frequency (at least one CpG island methylated) in comparison with people younger than 20 years (68.1 vs 50.5 %) (P=0.02). Specifically, we observed a significant increase in *LIG4* methylation with the age (0 vs 8.8 %; P=0.02). *XRCC5* methylation also showed an increase with the age (42.8 vs 57.1 %), although it did not reach the statistically significant value (P=0.05). In contrast, the frequency of *XRCC6* methylation was higher in individuals <20 years compared with older individuals (>40 years old) (9.9 vs 1.1 %; P=0.01). The rest of CpG islands analyzed showed no remarkable results (Fig. 2). Finally, when the presence of more than one CpG island methylated was studied, no differences between adults and young people were found (11 vs 13.2 %; P=0.06).

Among individuals with a translocation, the frequency of individuals >40 years old with at least one methylated CpG island was slightly higher than that obtained for the group of individuals younger than <20 years old (56.6 vs 50 %; P=0.6). Surprisingly, among individuals without translocations, the frequency of methylation was significantly higher among individuals older than 40 years old (80.4 vs 50.9 %; P=0.002) (Table 4). No differences in the frequency of methylation between samples with and without translocations were detected (53.2 vs 64.1 %; P=0.14).

Discussion

The present study shows that methylation of NHEJ pathway genes does not contribute significantly to the increase of translocations with the age. Although we observed a higher frequency of *Bcl2-JH* and *BCR-ABL* (*major*) translocations with aging and two NHEJ genes presented age-dependent promoter methylation changes, we did not observe a correlation between these two events.

Both translocations, *BCL2-JH* and *BCR-ABL*, are chromosome abnormalities characteristics of adult lymphomas. The *BCL2-JH* or t(14;18) translocation is a genetic hallmark of FL. In our study population, we detected that this rearrangement was present in 37.8 % of our population, similar to that described in other studies performed in healthy individuals (40–60 %) (Nambiar and Raghavan 2012). Moreover, the frequency of the translocation increased with the age of individuals (30.9 vs 46.2 %), results which were also in line with previous studies (Dölken et al. 2008; Liu et al. 1994; Nambiar and Raghavan 2010; Schüler et al. 2009). The chromosome translocation *BCR-ABL* or t(9;22) involving the major region of *BCR* is found in patients with chronic myeloid leukemia (CML) and adult acute lymphoblastic leukemia (Zhen and Wang 2013). This translocation was also detected in blood cells of 9.9 % healthy individuals of our study population. This frequency was lower than those previously reported (~20 %) by other authors (Biernaux et al. 1995; Bose et al. 1998). In addition, a significant increase of the frequency of the BCR-ABL translocation was detected with the age of individuals (4.8 % in <40 years old vs 14.7 % in >40 years old). In contrast, *MLL-AF4* or t(4;11) translocation, characteristic of pediatric acute lymphoblastic leukemia and uncommon in adult leukemia, was found to be more frequent in individuals younger than 40 years old than in adults (12 vs 1 %). These results can be explained by the mechanisms involved in the biogenesis of translocations, which include the spatial proximity of chromosomes (Parada and Misteli 2002). The proximity between *BCL2-JH* and *BCR-ABL* has been already demonstrated in lymphocytes of adults (Neves et al. 1999; Roix et al. 2003). Hence, the higher frequency of these translocations in

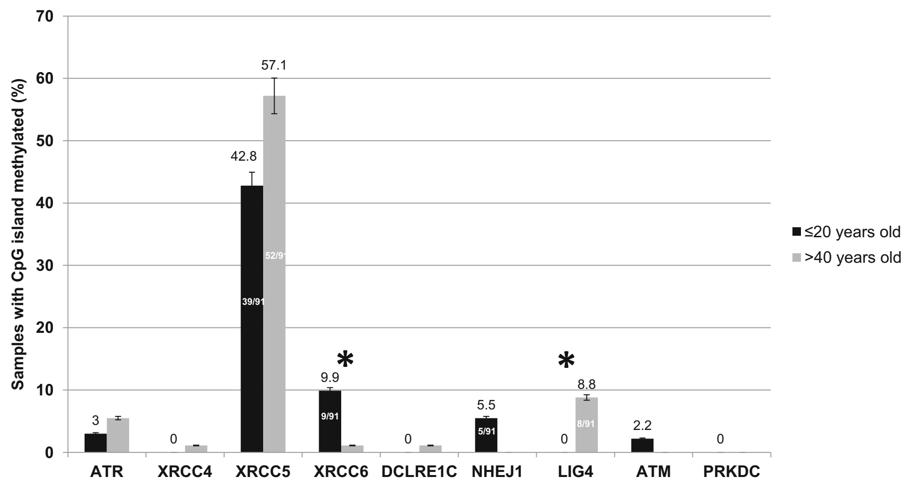

Fig. 2 Methylation-specific PCR results. The percentage of methylation in 9 CpG islands was compared in young people ≤20 years old vs adults >40 years old. *Asterisk* represents statistically significant results

adults could be due to the acquisition of de novo alterations with the age because of replication errors. However, *MLL-AF4* translocation occurs more frequently in children. The origin of pediatric cancers has been suggested to happen during the embryonic development (Brassesco 2008; Greaves 2003), so an explanation for the decrease of this rearrangement with the age could be that these loci are close during embryogenesis, but this proximity disappears in adults.

Nowadays, the biological significance of the occurrence of translocations in healthy people still remains unresolved, although a recent study has showed that the presence of *BCL2-IgH* translocation in blood from healthy individuals works as a predictive biomarker for FL, being effective years before diagnosis (Roulland et al. 2014). This means that the determination of translocations in healthy individuals could be a useful tool to predict the risk of developing some lymphomas or leukemias in the near future.

Table 4 Methylation status of 182 healthy individuals in relation to age and the presence of translocations

Age	Yes, number, (%)	No, number, (%)	*P* value[a]
Translocation	Methylation		
>40 years	25 (56.6)	20 (44.4)	0.6
≤20 years	17 (50.0)	17 (50.0)	
No translocation	Methylation		
>40 years	37 (80.4)	9 (19.6)	0.002
≤20 years	29 (50.9)	28 (49.1)	

[a] X^2 test was applied

To determine if the increase of *BCL2-JH* and *BCR-ABL* translocations with aging was the result of NHEJ pathway genes silencing through epigenetic mechanisms, we analyzed the methylation status of 11 NHEJ genes considering the frequency of translocations and the age. We found a higher number of individuals with the *LIG4* promoter methylated in the group of adults (>40 years old) compared with the younger group (≤20 years old) (8.8 vs 0 %). Remarkably, *LIG4* promoter was also found to be hypermethylated in different types of lymphomas (Martín-Subero et al. 2009). However, no analysis on healthy individuals had been done until now. *LIG4* forms together with *XRCC4* a complex that is responsible for the NHEJ final ligation step (Ahnesorg et al. 2006; Buck et al. 2006). The fundamental role of these two factors has been thoroughly demonstrated. Mutations in *LIG4* lead to variable immunodeficiency, developmental delay, chromosome alterations, and microcephaly (Buck et al. 2006; O'Driscoll et al. 2001). Of note is the fact that *LIG4* or *XRCC4* deficient mouse B cells showed decreased class-switch recombination and substantial levels of IgH locus chromosomal breaks (Yan et al. 2007). Therefore, we could speculate that *LIG4* promoter hypermethylation, observed in a higher frequency in adults, could lead to *LIG4* gene silencing in some cells, increasing the risk of translocations in this group. However, no significant results were observed when the frequency of methylation and translocation were analyzed together.

The *XRCC5* gene promoter was also found to be methylated in a higher number of healthy adults (57.1 vs 42.8 %, although not statistically significant). The deregulation of *XRCC5* due to the hypermethylation of its promoter was also observed in a subset of non-small cell lung cancer patients (Lee and McKinnon 2007). By contrast, the methylation of *XRCC6* promoter was significantly less frequent in the older group (1.1 vs 9.9 %). *XRCC5* and *XRCC6* encode for the ku80 and ku70 proteins, respectively, which form the Ku heterodimer. Alterations of this complex have been related to the decreased DNA repair capacity in the elderly (Doria et al. 2004; Ju et al. 2006; Seluanov et al. 2007). However, according to our results, the methylation changes observed in these two genes during aging seems to be not associated with an increase of translocations frequency.

In summary, in the present work, we show a higher frequency of two translocations with the age that cannot be explained by a loss of fidelity of NHEJ pathway with aging through the methylation of its genes. This suggests the involvement of other unknown molecular mechanisms.

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Conflict of interest All the authors declare no conflict of interest.

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