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Received December 26, 2019; Accepted May 19, 2020

DOI: 10.3892/ol.2020.11745

Abstract. DNA hypomethylation of long interspersed repetitive DNA retrotransposon (LINE-1) and Alu repeats elements of short interspersed elements family (SINEs) is an early event in carcinogenesis that causes transcriptional activation and leads to chromosomal instability. In the current study, DNA methylation levels of LINE-1 and Alu repeats were analyzed in tumoral tissues of invasive breast cancer in a Tunisian cohort and its association with the clinicopathological features of patients was defined. DNA methylation of LINE-1 and Alu repeats were analyzed using pyrosequencing in 61 invasive breast cancers. Median values observed for DNA methylation of LINE-1 and Alu repeats were considered as the cut-off (59.81 and 18.49%, respectively). The results of the current study demonstrated a positive correlation between DNA methylation levels of LINE-1 and Alu repeats (rho=0.284; P<0.03). DNA hypomethylation of LINE-1 was also indicated to be associated with low grade (P=0.023). To the best of our knowledge, the current study is the first study regarding DNA methylation of LINE-1 and Alu repeats element in breast cancer of the Tunisian population. The results of the current study suggest that, since hypomethylation of LINE-1 is associated with low grade, it could be used as a biomarker for prognosis for patients with breast cancer.

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Key words: breast cancer, DNA methylation, hypomethylation, long interspersed repetitive DNA retrotransposon, alu repeats, pyrosequencing

Introduction

Breast cancer is the most frequently diagnosed malignancy in women worldwide and the most common cause of death from cancer (1).

It is a heterogeneous disease caused by a combination of genetic, hormonal, and environmental factors. Moreover, epigenetic modifications have also been shown to be implicated in breast cancer progression. These alterations, including DNA methylation, are one of the emerging and promising research fields in human cancer (2), and are an early event in carcinogenesis and play a role as relevant as genetic mutations (3,4). DNA Methylation is a post replicated reversible molecular modification consisting of the addition of a methyl group (-CH3) to the 5' cytosine of the pyrimidine ring in the CG dinucleotide sequence known as CpG. This addition is mediated by enzymes of the DNA methyl-transferases (5). CpG dinucleotides represent 2-5% of the whole genome sequence and the majority of these sequences (60-80%) are methylated in normal tissues (6,7).

While the regional DNA hypermethylation has been well characterized in human cancer, this was not the case for the global DNA hypomethylation (8,9). The latter occurs in CpG poor regions and repetitive elements and is associated with genomic and chromosomal instabilities leading to the activation of oncogenes. DNA hypomethylation happens in about 50% of breast cancers (6,10) and correlates with histologic grade, stage and malignancy (11).

DNA repetitive elements are well represented and dispersed throughout the human genome and constitute about 50% of the human genome (12). There are two major repetitive elements sequences in the human genome. The first is the long interspersed repetitive DNA retrotransposon (LINE-1) composing about 17% of the human genome and present in over 500,000 copies. The second is the Alu repeats elements of short interspersed elements family (SINEs), composing 11% of the human genome and present with one million copies (13).

LINE-1 and Alu repeats are used as surrogate markers for global methylation status (14) because of their high abundance and density of CpG, 13% for LINE-1 and 23% for Alu repeats. In normal tissues, LINE-1 and Alu repeats are mostly heavily methylated. However, it has been observed that the DNA methylation levels of LINE-1 and Alu repeats could be different in some tissue types and genome locations (15-17).

In cancer cells, the hypomethylation of LINE-1 and Alu repeats have been described and associated with transcriptional enhancement (18,19), which may lead to genome instability and retrotransposition of transposable elements (20,21). Various studies reported that LINE-1 and Alu repeats were hypomethylated in different types of cancers such as colorectal (22), ovarian (23) and breast cancers (24,25).

Moreover, it was shown that the levels of LINE-1 and Alu repeats methylation change during breast cancer progression (26) and was associated with poor prognosis, poor clinical outcomes and also with tumor progression (20,27,28). The hypomethylation levels of LINE-1 and Alu repeats were observed as an early event in carcinogenesis (29,30). Based on several studies, it was suggested to use the hypomethylation of LINE-1 and Alu repeats as an epigenetic cancer biomarker for cancer diagnosis and prognosis (31,32).

This study aims to analyze the methylation levels of LINE-1 and Alu repeats in tumoral tissues of breast cancer using pyrosequencing in order to define the association of clinicopathological features with DNA hypomethylation in breast cancer in a cohort of Tunisian women.

Materials and methods

Study population. 61 breast cancer patients with primary invasive ductal carcinoma (IDC), who had undergone surgery between 2008 and 2010, at Salah Azeiz institute Tunis Tunisia, were enrolled in this study. Clinicopathological data were available for 60 patients and summarized in Table I. The study was approved by the Ethical Committee of Salah Azeiz Institute of Tunis and informed consent was obtained from all patients.

DNA isolation. DNA was extracted from fresh frozen tumoral tissue using DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. The quantity, as well as the quality of the extracted DNAs, were measured using both of the Nanodrop spectrophotometers by the A260/A280 ratio (Thermo Fisher Scientific, Inc.) and the Qubit 3 fluorometer (Life Technologies).

Methylation analysis. For the current study, genomic DNA was treated with sodium bisulfite conversion using the EpiTect 96 Bisulfite kit (Qiagen) according to the manufacturer's instructions. After bisulfite conversion, PCR reaction was performed with 20 ng of bisulfite-treated DNA in a 25 μl volume reaction which contained: 2.5 μl of the buffer, 1.6 μl (25 mM) of Mgcl2, 1 μl (10 mM) of dNTP, 0.2 μl of HotStar Taq DNA polymerase, 1 μl of bisulfite converted DNA and completing to 25 μl with H_2O. Regarding the LINE-1 primers, 0.5 μl (10 μM) forward and reverse primer were added, concerning the Alu repeats primers we used: 0.1 μl (10 μM) forward primer, 1 μl (10 μM) reverse primer, 0.9 μl (10 μM) Universal Biotin. PCR conditions were 10 min at 95˚C; 30 sec at 95˚C, 30 sec at the annealing temperature (Ta), 15 sec at 72˚C for 50 cycles; 5 min at 72˚C. The Ta for LINE-1 and Alu were 60˚C and 54˚C, respectively. The design of the sequences of the primers, listed in Table II, was based on previous studies (25,33).

Table I. Clinicopathological characteristics of the cohort (61 patients).

Characteristics	No. (%)
Age	
Mean	52.62
Range	33 to 81
Menopausal status	
Premenopausal	28 (45.9)
Postmenopausal	32 (52.5)
Not determined	1
Tumor size	
T1	5 (8.2)
T2	43 (70.5)
T3	4 (6.6)
T4	7 (11.5)
Not determined	2 (3.3)
Nodal status	
N0	10 (16.4)
N1	46 (75.4)
N2	3 (4.9)
Not determined	2 (3.3)
Pathological stage	
I	3 (4.9)
II	45 (73.8)
III	11 (18.0)
Not determined	2 (3.3)
Histologic grade	
I	9 (14.8)
II	34 (55.7)
III	16 (26.2)
Not determined	2 (3.3)
Oestrogen receptor status	
Negative	20 (32.8)
Positive	39 (63.9)
Not determined	2 (3.3)
Progesteron receptor status	
Negative	21 (34.4)
Positive	38 (62.3)
Not determined	2 (3.3)
HER2 amplification	
Negative	31 (50.9)
Positive	25 (41)
Not determined	5 (8.2)

HER2, human epidermal growth factor receptor 2.

Table II. Sequences of Primers used for LINE-1 and Alu repeats amplification.

Gene	Primer sequence 5'-3'	Number of CpGs	(Refs.)
Alu	Forward: 5'-GGGACACCGCTGATCGTATATTTTTATTAAAA ATATAAAAATTAGT-3'	4	(25)
	Reverse: 5'-CCAAACTAAAATACAATAA-3'		
	Universal Biotin primer: 5-Biotin-GGGACACCGCTGATCGTATA-3		
	Pyro primer: 5'-AATAACTAAAATTACAAAC-3'		
LINE-1	Forward: 5'-TTTTGAGTTAGGTGTGGGATATA-3'	5	(33)
	Reverse: 5'-Biotin-AAAATCAAAAAATTCCCTTTC-3'		
	Pyro primer: 5'-GGGTGGGAGTGAT-3'		

LINE-1, long interspersed repetitive DNA retrotransposon; CpG, 5'-Cytosine-phosphate-guanine-3'.

5 μl of the PCR reaction and no-template control were analyzed by electrophoresis on a 2% agarose gel. Pyrosequencing was performed to measure methylation levels of LINE-1 and Alu repeats. The biotinylated PCR products were bound to beads and the biotinylated strand was isolated as described in the Busato *et al* study (34). DNA methylation analysis was performed on a PyroMark Q96 MD (Qiagen) using PyroMark Gold SQA Q96 kit (Qiagen) according to the manufacturer's protocol. Data obtained was analyzed by PyroMark CpG Software, at each CpG site analyzed, the ratio of C to T nucleotides was calculated as a percentage for LINE-1 methylation, and the ratio of G to A was calculated as a percentage for Alu repeats methylation (20). The means methylation levels for 5 measured CpG sites of LINE-1 and for 4 measured CpG sites of Alu repeats were used for statistical analysis.

Statistical analysis. All statistical analysis were performed using the IBM SPSS Statistics for Windows software, v20 (IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

Pearson and Spearman correlation analysis were used to determine the correlation between methylation levels of LINE-1 and Alu repeats. The cut-off used to categorize samples as methylated or hypomethylated was the median values of the DNA methylation levels of LINE-1 and Alu repeats. Chi-square and Fisher's exact test were used to evaluate the associations between LINE-1 and Alu repeats methylation status and clinicopathologic characteristics. ANOVA followed by Tukey's multiple comparison post-hoc test were used to compare the means between grades. The disease-free survival (DFS) rate was analyzed by the Kaplan-Meir method and compared using the log-rank test.

Results

The methylation levels of two repetitive elements LINE-1 and Alu repeats were analyzed to evaluate DNA hypomethylation in tumor tissue from 61 breast cancer patients. Clinicopathological data are summarized in Table I. The mean age of the patients was 52.62 years. Correlation analysis revealed a weak positive correlation between LINE-1 and Alu repeats DNA methylation levels (Pearson coefficient, rho=0.284, P<0.03), (Spearman coefficient, rho=0.341, P=0.009) (Fig. 1). We performed the correlation test between LINE-1 and Alu repeats DNA methylation levels separately in the Grade I, Grade II, and Grade III. As results we obtained that there was no correlation between line 1 and Alu in: Grade I (Pearson coefficient, rho=0.384, P=0.307), (Spearman coefficient, rho=0.391, P=0.134), Grade II (Pearson coefficient, rho=0.256, P=0.164), (Spearman coefficient, rho=0.340, P=0.062). Grade III (Pearson coefficient, rho=0.304, P=0.252) (Spearman coefficient, rho=0.100, P=0.798).

The distribution of the methylation levels for both LINE-1 and Alu repeats is illustrated in (Fig. 2). The median methylation level was 59.81% for LINE-1 (from 29.66 to 74.12%, mean 58.12%) and was 18.49% (from 15.75 to 24.22%, mean 18.38%) for Alu repeats. The median was considered as the cut-off for LINE-1 and Alu repeats DNA hypomethylation. The association between clinicopathological parameters and DNA methylation levels (<median vs. >median) of both repetitive elements is shown in Table III. LINE-1 DNA hypomethylation was significantly associated with low grade (P=0.023). The median methylation level of LINE-1 in high-grade breast cancer was 62.41%, while low grade was 59.08%. Box plot comparing the Grade I, Grade II and Grade III according to LINE-1 methylation levels (A) and according to Alu methylation levels (B) is illustrated in the (Fig. 3). We performed the ANOVA test comparing the means between grades, obtaining a p value of P=0.382. We then used Tukey's test as a post-hoc test, obtaining as results: (Grade 1-Grade 2) P=0.646, (Grade 1-Grade 3) P=0.351, (Grade 2-Grade 3) P=0.712. These results are not statistically significant.

Furthermore, no statistically significant associations were found for DNA hypomethylation of LINE-1 with other parameters including patient's age (P=0.215), T stage (P=0.173), N stage (P=0.570), ER status (P=0.534) and PR status (P=0.579), HER2 status (P=0.206). There was no significant associations for DNA hypomethylation of Alu repeats with all clinicopathological parameters: Age (P=0.551), T stage (P=0.416) N stage (P=0.589), ER status (P=0.320), PR status (P=0.231) HER2 status (P=0.561) and grade (P=0.552).

We analyzed patient disease-free survival curve in 45 patients by Kaplan-Meier analyses, the median follow-up time was 69,95 months (range 1.91 to 131,99) median was chosen as a cut-off to define LINE-1 and Alu repeats hypomethylation (<59.81%, <18.49%). There were six distant

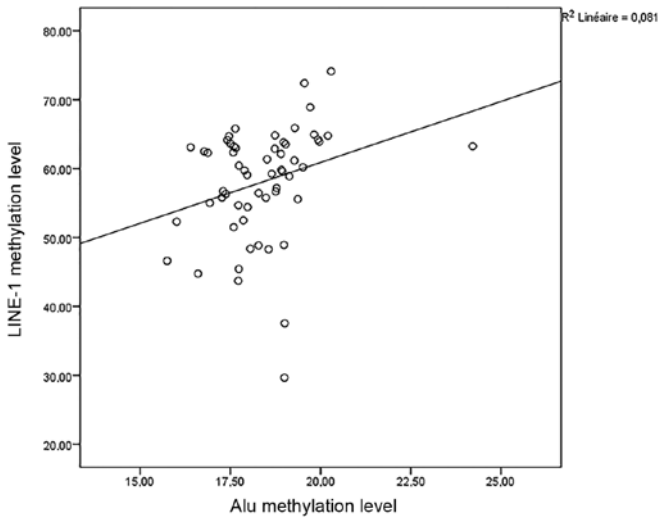

Figure 1. Scatter plot illustrating the weak positive correlation between LINE-1 and Alu DNA methylation levels (Pearson coefficient, rho=0.284; P<0.03). LINE-1, long interspersed repetitive DNA retrotransposon.

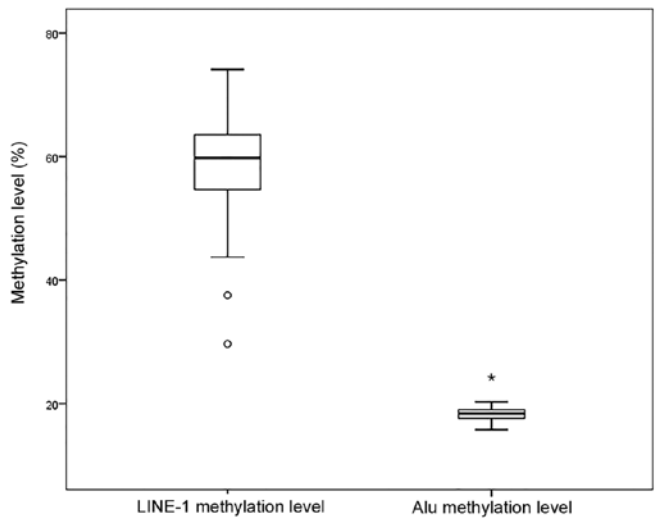

Figure 2. Box plot illustrating the variation of methylation of LINE-1 (median methylation level was 59.81%), and of Alu repeats (median methylation level was 18.49%). LINE-1, long interspersed repetitive DNA retrotransposon.

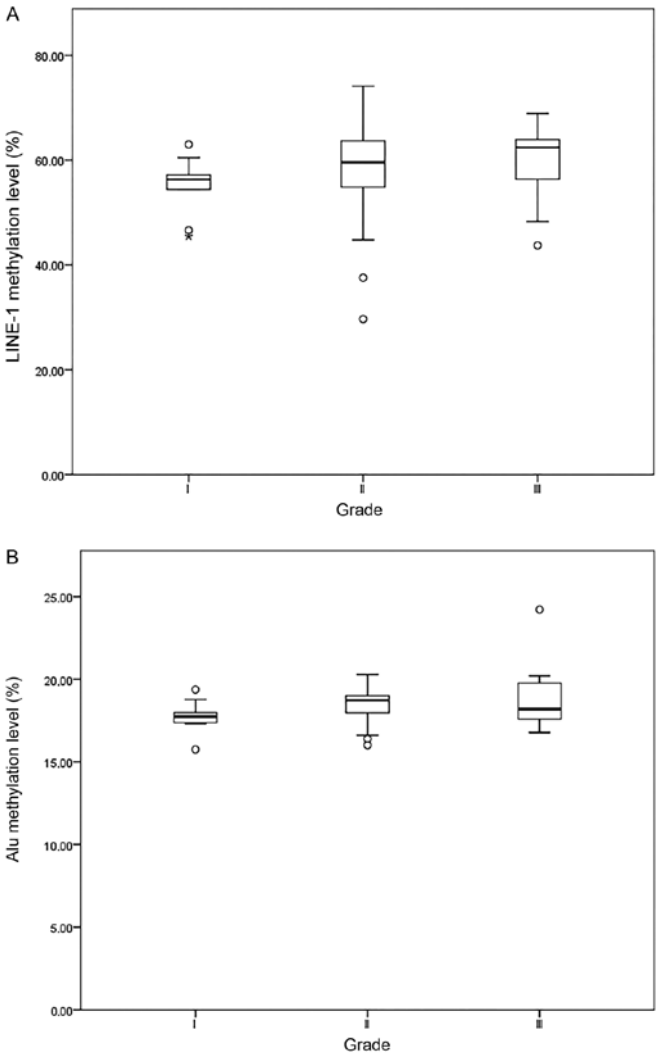

Figure 3. Box plot comparing the Grade I, Grade II and Grade III according to (A) LINE-1 methylation levels and according to (B) Alu methylation levels. LINE-1, long interspersed repetitive DNA retrotransposon.

metastases (2.7%) and one loco-regional recurrence (0.45%). LINE-1 and Alu repeats hypomethylation were not associated with shorter disease-free survival time (log-rank test, P=0.312 and P=0.632), respectively.

Discussion

Currently, several different classifications to determine the prognosis in breast cancer patients, based on histopathological criteria and immunohistochemistry results are used but have shown some limits due to the heterogeneous nature of the disease (35,36). DNA methylation markers have been suggested as a new and promising approach for the stratification of the patients in cancer that could bear some prognosis and predictive information (37).

The purpose of our study was to evaluate the DNA methylation levels of the repetitive elements LINE-1 and Alu repeats in tumoral breast cancer cells, which were never studied in Tunisian patients and to investigate for possible associations with clinicopathological features. The measurement of DNA methylation level was performed by pyrosequencing. This sequencing-by-synthesis method stands out from other techniques by offering us a considerable advantage through the high quality and the quantitative nature of the results (38,39).

LINE-1 and Alu repeats are crucial contributors to the dynamics, plasticity and integrity of the human genome (40). A previous study suggests that the hypomethylation of these repeats elements might contribute to a significant portion of the development and progression of cancer by activating them (41). This mechanism leads to recombination events and insertions (42).

In the present study, we found that methylation of LINE-1 and Alu repeats correlates positively The status of the two patients who give a very high hypomethylation of LINE according to (Fig. 1) is Grade II for both of them, which explains the low correlation between methylation levels. Since LINE-1 and Alu repeats are dispersed throughout the whole genome. They can be considered as being a surrogate marker for global

Table III. Clinicopathological characteristics of patients according to LINE-1 and Alu methylation status of invasive breast cancer.

Characteristic	Alu hypomethylated <median (18.49%)	Alu methylated ≥median (18.49%)	P-value	LINE-1 hypomethylated <median (59.81%)	LINE-1 methylated ≥median (59.81%)	P-value
Number (%)	30 (50)	30 (50)		29 (49.2)	30 (50.8)	
Age at diagnosis (Row %)			0.551[b]			0.215[b]
<50 years	13 (46.43)	15 (53.57)		16 (57.14)	12 (42.86)	
≥50 years	16 (51.61)	15 (48.39)		12 (40)	18 (60)	
Not determined	1	-		2	-	
Tumor size (Row %)			0.416[a]			0.173[a]
T1	1 (20)	4 (80)		1 (25)	3 (75)	
T2	22 (51.16)	21 (48.84)		23 (54.76)	19 (45.24)	
T3 and T4	5 (45.45)	6 (54.54)		3 (27.27)	8 (72.72)	
Not determined	2	-		-	-	
Nodal status (Row %)			0.589[b]			0.570[b]
Positive	5 (50)	5 (50)		4 (44.4)	5 (55.5)	
Negative	23 (47.91)	25 (52.09)		23 (47.91)	25 (52.08)	
Not determined	2	-		1	-	
Pathological stage (Row %)			0.228[a]			0.601[a]
I	0 (0)	3 (100)		1 (33.33)	2 (66.66)	
II	23 (51.11)	22 (48.89)		2 (51.16)	21 (48.84)	
III	5 (50)	5 (50)		4 (36.36)	7 (63.64)	
Not determined	2					
Histologic grade (Row %)			0.552[b]			0.023[b]
Low grade (I/II)	20 (47.62)	22 (52.38)		24 (58.53)	17 (41.46)	
High grade (III)	8 (50)	8 (50)		4 (25)	12 (75)	
Not determined	2	-		-	1	
ER			0.320[b]			0.534[b]
Negative	11 (57.9)	8 (42.1)		10 (50)	10 (50)	
Positive	18 (46.15)	21 (53.85)		18 (47.37)	20 (52.63)	
Not determined	1	1		1	-	
PR			0.231[b]			0.579[b]
Negative	12 (60)	8 (40)		10 (47.62)	11 (52.39)	
Positive	17 (44.74)	21 (55.26)		18 (48.65)	19 (51.36)	
Not determined	1	1		1	-	
HER2 amplification			0.561[b]			0.206[b]
Negative	15 (48.39)	16 (51.61)		17 (56.66)	13 (43.33)	
Positive	12 (50)	12 (50)		10 (41.66)	14 (58.33)	
Not determined	3	2		2	3	

[a]χ^2 test; [b]Fisher's exact test; ER, Oestrogen receptor status; PR, progesterone receptor status; LINE-1, long interspersed repetitive DNA retrotransposon.

methylation status. Moreover, Ross *et al* (43) reported in their study that repeats elements hypomethylation, as well as demethylation of single-copy genes, are mechanistically linked and might be mediated by the same factors. Thus, we suggest that some oncogenes may also be hypomethylated in our cohort.

Furthermore, based on several previous studies on various types of tissues, we applied the median value as a cut-off for the methylation levels (hypomethylated vs. methylated) for both LINE-1 and Alu repeats. Our median value was similar to large scale studies values (20,26,44). The median value of our cohort for Alu repeats was 18.49%. Previous studies suggested that the cut-off median for Alu repeats were 20.2% in breast cancer. The median value for LINE-1 was 59.81% which was close to the selected cut-off median for LINE-1

59.4% in breast cancer in former studies (26). We observed a great variation of LINE-1 methylation levels, which is concordant with the literature (26). Furthermore, unlike LINE-1, we observed the stability of Alu methylation levels. Moreover, the methylation levels of repeated elements are relative to the elements by themselves. Therefore, a relatively low methylation of LINE-1 should be considered as hypomethylation even though it is superior to the Alu median level of methylation.

Moreover, it was reported that, in several carcinomas such as breast and colon cancer, there is a great difference in methylation levels of LINE-1 and Alu repeats between tumoral tissues and their adjacent normal tissues (28,45,46). Previous studies have shown that DNA methylation levels of LINE-1 were from 70 to 90% in normal tissues (47,48), and were from 55 to 60% in tumor tissues. These results suggest that patients with cancer could be characterized by global hypomethylation (49,50). Regarding Alu repeats methylation levels, it was reported that in normal somatic tissue, this element was highly methylated (51,52). In cancer tissues, earlier studies have shown that the level of hypomethylation of Alu repeats was different among cancer types. In breast cancer, there is a lower level of hypomethylation than in colon and lung cancer, which exhibited a higher level of hypomethylation (30).

Our results showed that the hypomethylation of LINE-1 was associated with low-grade tumors, while it has been suggested that DNA mutations are associated with high-grade tumors. The mechanism by which LINE-1 hypomethylation affects tumor grade remains unclear. We could suggest that the LINE-1 hypomethylation, which leads to chromosomal instability (49), might represent one of the possible mechanisms. On the other hand, the antisense promoter of LINE-1 can activate the gene expression of surrounding genes (53), which might have been involved in tumor grading; this could also be a hypothesis explaining the mechanism. Given that LINE-1 hypomethylation is associated with low grade, it could be considered as a good prognostic marker. In that way, methylation analysis is not superior to histologic studies, but it should be used in combination with histologic analysis.

Moreover, no statistically significant associations were found for hypomethylation of LINE-1 with clinicopathological features ER, PR, HER2 status, age, T stage and N stage. Regarding the hypomethylation of Alu, no statistically significant associations were found with all clinicopathological features. Different results were obtained in various studies of breast cancer. For example, Park *et al* (26) showed that hypomethylation of LINE-1 was associated with negative ER status, negative PR status, and positive HER2 status. However, Alu repeats hypomethylation was only associated with ER negativity. van Hoesel *et al* (24), reported that in breast cancer, LINE-1 hypomethylation was associated with tumor stage, nodal status and age. Cho *et al* (54) showed that LINE-1 hypomethylation was statistically associated with premenopausal women, whereas the methylation level of Alu repeats was not associated with any clinical outcome.

The relationship between overall survival or disease-free survival and DNA hypomethylation of LINE-1 and Alu repeats have been analyzed in various studies in human cancer (20,55). In the present study, LINE-1 and Alu repeats hypomethylation were not associated with shorter disease-free survival. Park *et al* (26) reported that LINE-1 hypomethylation was also not associated with disease evolution, whereas Alu hypomethylation tended to be associated with poor disease-free survival, van Hoesel *et al* (24) reported that in young breast cancer patients (≤55 years), LINE-1 hypomethylation was associated with a bad prognosis.

It seemed difficult to compare results from several studies since they were based on different sample sizes varying from small to a large cohort. Several low throughput methods AQAMA PCR assay (24), Methylight (54), pyrosequencing (56), have been developed and used in studies for the analysis of DNA methylation. However, none of them appeared to be the 'gold-standard' technique that combines high sensitivity, quantitative accuracy and cost-effectiveness. In various studies, several experimental designs were proposed, different consensus sequences were chosen to analyze DNA methylation levels of repetitive elements, without reaching a common experimental design. Moreover, there was no optimal consensual cut-off. Park *et al* (26) applied the median value as a cut-off, whereas van Hoesel *et al* (24) used the 25th percentile of methylation levels to define the hypomethylated group; other studies were based on a qualitative method to identify the presence of methylation.

The limitation of our study is the number of methylated samples. Nevertheless, considering the small size of the Tunisian population (11.78 millions), our cohort of 61 patients could be considered informative to determine a decrease of methylation frequency of LINE-1 and Alu repeats.

Based on the results of several studies, it was suggested to use LINE-1 as a surrogate marker for global methylation status and potentials biomarker for negative prognostic and breast cancer risk (24,57). In this first study regarding DNA methylation of LINE-1 and Alu repeats element in breast cancer of the Tunisian population, our results showed a positive correlation between DNA methylation levels of LINE-1 and Alu repeats. Regarding association with clinical features, DNA hypomethylation of LINE-1 was associated with low grade, which suggests that LINE-1 hypomethylation could be used as a biomarker for good prognostic.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

HRZ, AH, HD, JFD and MK designed the experiments. HRZ performed experiments and wrote the original draft. HRZ, AH and AD analyzed data. IB, OA and AG performed immunohistological investigation. KR performed clinical investigation. HRZ and MS performed statistical analysis of the data. AH and MK performed validation of the study, and

reviewed and edited the final manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethical Committee of Salah Azeiz Institute of Tunis. Informed consent was obtained from all patients.

Patient consent for publication

Informed consent was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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