Int J Clin Exp Pathol 2014;7(7):4104-4111
www.ijcep.com /ISSN:1936-2625/IJCEP0000359

Original Article

Decreased LINE-1 methylation levels in aldosterone-producing adenoma

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Received March 27, 2014; Accepted June 23, 2014; Epub June 15, 2014; Published July 1, 2014

Abstract: Purpose: Abnormal global DNA methylation levels are associated with many diseases. In this study, we examined long interspersed nuclear elements-1 (LINE-1) methylation as a biomarker for abnormal global DNA methylation and aldosterone-producing adenoma (APA). Methods: Tissues from 25 APA and 6 normal adrenal glands (NAs) were analyzed for LINE-1 methylation by real-time methylation-specific polymerase chain reaction. The estimated LINE-1 methylation level was then tested for correlation with the clinicopathologic parameters of APA patients. Results: The methylation index (MI) level for LINE-1 was 0.91 in NA samples and 0.77 in APA samples ($P < 0.001$). For the APA samples, there were no statistical correlations between the MI level and various clinicopathologic parameters such as gender ($P = 0.07$). Conclusion: LINE-1 methylation is significantly lower in APA samples than in NA samples. LINE-1 methylation is not correlated with the clinical characteristics of APA.

Keywords: Long interspersed nuclear elements-1, global DNA methylation, aldosterone-producing adenoma

Introduction

Primary aldosteronism (PA) is the most prevalent form of endocrine hypertension [1]. PA results from autonomous aldosterone secretion, which then leads to hypertension with hypokalemia and suppressed renin activity. The two major subtypes of PA are unilateral aldosterone-producing adenoma (APA) and bilateral adrenal hyperplasia, which together account for approximately 95% of cases [2]. Although some evidence indicates that a subset of PAs arise from somatic mutations in the gene encoding the selectivity filter of the KCNJ5 K+ channel [3], the ATP1A1 gene encoding the Na+/K+ ATPase α subunit and the ATP2B3 gene encoding a Ca2+ ATPase [4], the pathophysiological mechanisms resulting in APA and the development of bilateral adrenal hyperplasia are still not well understood.

Increasing evidence indicates that epigenetic events, such as genome-wide losses in DNA methylation, frequently occur in malignancies and may be critical in carcinogenesis [5, 6]. Loss of DNA methylation is also associated with other disease states including stroke and heart disease [7]. As there are critical links between genomic hypomethylation and pathogenesis, there is growing interest in understanding how changes in the global status of DNA methylation can be used as biomarkers for diseases such as APA.

Long interspersed nuclear elements (LINE-1) are non-long terminal-repeat (non-LTR) retrotransposons that make up approximately 17% of the human genome, with 500,000 elements normally dispersed throughout the human genome [8, 9]. LINE-1 sequences are frequently repeated and widely interspersed human retrotransposons; therefore, their methylation levels can mark changes in global genomic DNA methylation [10]. In this study, we evaluated LINE-1 methylation status in APA.

Materials and methods

Study subjects

This hospital-based study on APA (25 cases) was conducted in the Department of Endo-

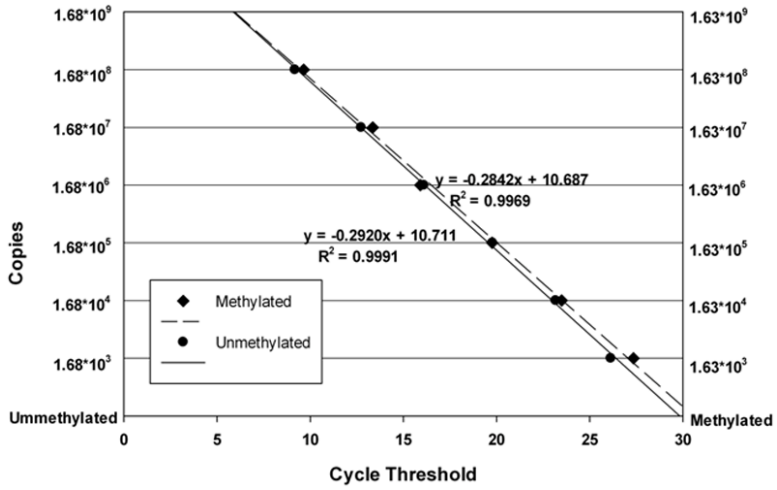

Figure 1. Standard curves for the methylated and unmethylated LINE-1 reactions.

crinology, Zhongshan Hospital. The cases, which were clinically diagnosed and histologically confirmed according to the guidelines of the Endocrine Society [11], were from patients aged 31 to 70 years (mean, 48 ± 11 years) recruited between 2009 and 2013. All of the recruited patients were Chinese. We used the plasma aldosterone-renin ratio (ARR) for case detection (ARR > 40; plasma aldosterone concentration (PAC) (ng/dl) / plasma renin activity (PRA) [ng/(ml·h)]). All patients showed positive results for at least one of the confirmatory tests, which included the captopril challenge test and the saline infusion test. These tests, as well as the follow-ups, were performed after the patients had discontinued antihypertensive treatment for at least 2 weeks to minimize the interference of medication with the PAC and PRA measurements. In this study, hypokalemia was designated as serum potassium concentrations less than 3.5 mmol/l. Adrenal venous sampling (AVS) with ACTH stimulation was performed for all APA diagnoses. The AVS was considered to show a unilateral lesion when the lateralized ratio (LR; high to low side) was greater than 4. Unilateral laparoscopic adrenalectomy was performed in the patients with APA. After the surgery, the hypokalemia was cured in 100% of cases. In addition, the hypertension was either cured or increasingly improved in 100% of cases. The control group (6 cases) was composed of sudden death patients. The 25 APA tissues and 6 normal adrenal (NA) tissues (the adrenal medulla was dissected out) were immediately collected and stored at -196°C in liquid nitrogen.

This study was approved by the Zhongshan Hospital ethics committee. All study subjects provided written informed consent.

Quantification of LINE-1 methylation levels

The DNA from the frozen tissues was isolated using a conventional proteinase-K organic extraction method, as previously described [12]. The frozen pulverized powders containing the genomic DNA were re-suspended in 0.4 ml lysis buffer (10 mM Tris-HCl pH 7.5, 20 mM EDTA pH 8.0, 0.5% SDS and 100 mM NaCl). Proteinase K (Tiangen, Shanghai, China) was added to the cellular lysates at a final concentration of 200 μg/ml, and the digestion was carried out at 55°C for 3-5 h. Organic extractions were performed with a half volume of phenol/chloroform/isoamyl alcohol (1:1:0.04). The extractions were repeatedly carried out until no visible interphase materials remained after centrifugation. The DNA was precipitated from the aqueous phase in the presence of a one-third volume of 7.5 M NH4Ac and three volumes of ethanol. The DNA pellet was washed once with 75% ethanol and dissolved at 65°C for 10 min with 0.2-0.4 ml TE (10 mM Tris-HCl pH 7.4 and 1 mM EDTA). The DNA was then stored at -20°C until further use. The DNA concentrations were calculated according to their OD 260 nm readings. Sodium bisulfite conversion was performed according to Axel Schumacher's protocol (http://www.methylogix.com/genetics/protocols.shtml-Dateien/schumachersguide1.html). The level of LINE-1 methylation was measured with a methylation-specific real-time polymerase chain reaction (PCR) assay [13, 14]. The primers used were as follows:

LINEa: unmethylated LINE-1 forward primer, TGTGTGTGAGTTGAAGTAGGGT; LINEb: unmethylated LINE-1 reverse primer, ACCCAATTTTCCAAATACAACCATCA; LINEc: methylated LINE-1 forward primer, CGCGAGTCGAAGTAGGGC; and LINEd: methylated LINE-1 reverse primer, ACCCGATTTTCCAAATACGACCG.

The plasmid pTA/LINEa-d consisting of the unmethylated and methylated LINE-1 amplicon was generated for use as a constant reference.

Table 1. MI values and clinicopathologic parameters of the APA tissues

No	Gender	Age	MI	ARR	Captopril challenge test	Saline infusion test	AVS Left: right
1	Female	47	0.75	189		+	4:1
2	Female	31	0.86	48		+	4:1
3	Female	34	0.83	336		+	1:8
4	Male	51	0.79	192		+	1:4
5	Female	51	0.76	236		+	1:6
6	Male	39	0.75	148		+	1:7
7	Male	69	0.83	192		+	1:4
8	Male	54	0.76	74		+	4:1
9	Female	30	0.74	178		+	1:5
10	Male	70	0.70	62	+		5:1
11	Male	36	0.81	386	+		9:1
12	Female	59	0.85	200		+	1:5
13	Female	59	0.73	209		+	8:1
14	Male	46	0.77	52		+	1:4
15	Male	33	0.79	183		+	6:1
16	Male	43	0.80	187		+	1:5
17	Male	49	0.75	511		+	9:1
18	Male	57	0.78	189	+	+	6:1
19	Female	48	0.81	141		+	1:6
20	Male	38	0.72	55		+	1:5
21	Male	55	0.73	289		+	5:1
22	Male	38	0.71	137		+	5:1
23	Male	59	0.73	168	+		1:5
24	Male	45	0.69	188	+	+	1:4
25	Female	51	0.79	284	+		1:6

No	Diameter (cm)	Pre-surgery			Post-surgery		
		MAP (mmHg)	K+ (mmol/l)	PAC (ng/dl)	MAP (mmHg)	K+ (mmol/l)	PAC (ng/dl)
1	1.5	133	2.5	250.0	100	3.5	170.9
2	1.3	128	2.6	301.0	94	4.1	89.0
3	1.1	120	2.0	335.3	96	3.5	98.4
4	1.0	130	2.0	199.2	98	3.6	98.2
5	1.3	146	2.6	305.0	120	3.7	111.6
6	0.6	138	3.1	234.0	136	4.0	143.7
7	1.0	133	2.7	243.0	110	3.4	154.5
8	1.8	133	1.9	162.8	112	3.7	98.0
9	1.0	147	2.8	231.9	95	3.4	100.0
10	1.0	97	2.5	246.6	98	3.7	97.2
11	1.6	160	2.1	193.1	95	3.7	98.0
12	1.6	102	2.0	266.0	103	3.7	121.7
13	1.1	133	2.7	250.6	133	4.0	134.4
14	1.0	143	3.2	204.2	130	3.9	133.6
15	1.5	133	2.7	257.0	94	3.4	100.2
16	1.0	120	2.1	276.0	95	3.7	94.7
17	1.6	147	1.8	306.6	96	3.5	89.9
18	2.0	119	3.3	152.6	110	3.4	99.1
19	1.0	123	3.0	323.1	97	3.7	112.6

20	1.4	149	1.9	253.5	97	3.5	124.8
21	1.2	133	2.3	231.4	118	3.8	160.7
22	1.5	157	3.1	383.6	95	3.9	133.6
23	1.7	130	3.6	218.3	97	4.1	89.7
24	1.5	144	3.2	195.0	94	3.6	96.8
25	1.1	132	1.8	251.0	125	3.4	87.2

Table 2. MI values and basic information of NAs

No	Gender	Age	MI
1	Female	47	0.93
2	Male	66	0.89
3	Male	20	0.89
4	Male	51	0.92
5	Female	51	0.91
6	Male	39	0.90

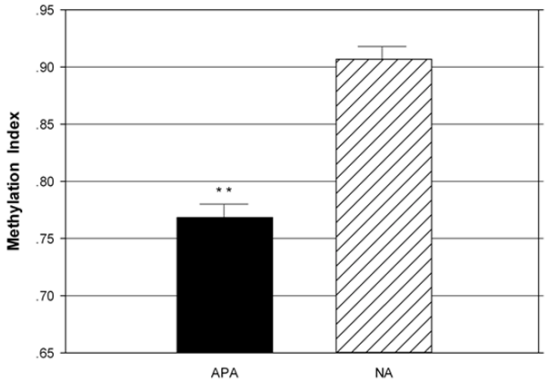

Figure 2. Methylation index (MI) of LINE-1 in the APA and NA tissues (**P < 0.001).

The PCR products of the unmethylated and methylated LINE-1 sequences were cloned by the pGEM-T Easy Vector system (Tiangen) and designated as pTA/LINEa-b and pTA/LINEc-d, respectively. This plasmid pTA/LINEa-d was used as a standard for the measurement of the unmethylated and methylated LINE-1 sequences. Serial dilutions of this plasmid were used to produce accurate and reproducible results. Real-time PCR was conducted with an Opticon Monitor 3 system (Bio-Rad) using the SYBR Green Real-time PCR Master Mix (Toyobo, Code: QPK-201). The real-time reactions for the unmethylated and methylated LINE-1 sequences were performed simultaneously in one 96-well plate. The methylation index (MI) for LINE-1 was calculated according to 100 × methylated *reaction/(unmethylated reaction + methylated reaction)*.

Statistical analysis

All statistical analyses were performed using SPSS software (SPSS version 17.0). The LINE-1 methylation levels were estimated by independent sample t-tests with MI as a continuous variable. The interactions between LINE-1 methylation and the various clinical parameters were determined by Pearson's correlation. A probability of less than 0.05 was considered statistically significant.

Results

Quantitative methylation levels of LINE-1

We used Opticon Monitor 3 software to compose the standard curves for the methylated and unmethylated LINE-1 reactions (**Figure 1**).

The MI of LINE-1 was calculated according to the standard curves and listed in **Tables 1** and **2**. The mean MIs for LINE-1 were 0.77 (mean, 0.77 ± 0.05) for APA and 0.91 (mean, 0.91 ± 0.01) for NA. The LINE-1 MI was significantly lower for the APAs than for the NAs (P < 0.001) (**Figure 2**). The MIs analyzed according to the Independent Samples T-test protocol (http://lap.umd.edu/psyc200/handouts/psyc200_08-12.pdf).

LINE-1 methylation levels and clinicopathologic parameters of APA

The MI values and clinicopathologic parameters for APA are listed in **Table 1**. Based on the statistical analyses, for APA patients, we estimated the correlation of LINE-1 methylation with various clinicopathologic parameters, including gender, age, tumor diameter, maximum mean arterial pressure (MAP), ARR, and basic PAC (**Tables 3** and **4**). The MI was tended to be higher in males than in females, but no significant differences were noted (P = 0.07) (**Figure 3**). There were also no significant linear associations between MI and any of the clinicopathologic parameters.

Table 3. Linear correlations of the clinical parameters and MI values of LINE-1

		Age	ARR	Diameter	Pre-surgery			Post-surgery		
					MAP	K+	PAC	MAP	K+	PAC
MI	Coefficient	-0.14	0.13	-0.06	-0.31	-0.30	-0.31	-3.0	-1.1	-2.3
	P-value*	0.52	0.55	0.77	0.14	0.15	0.14	0.14	0.61	0.26

*Correlation is significant at the 0.05 level (2-tailed) test.

Table 4. Clinical parameters of APA

Parameter	Age (y)	ARR	Diameter (cm)	Pre-surgery			Post-surgery		
				MAP (mmHg)	K+ (mmol/l)	PAC (ng/dl)	MAP (mmHg)	K+ (mmol/l)	PAC (ng/dl)
N	25	25	25	25	25	25	25	25	25
Mean	47	193.36	1.30	133.20	2.50	250.83	105.52	3.68	113.54
SD	11	107.75	0.33	14.86	0.53	54.12	13.69	0.22	24.48
P-value*	0.96	0.96	0.51	0.67	0.58	0.76	0.54	0.41	0.56

*P < 0.05, the parameter is not normally distributed.

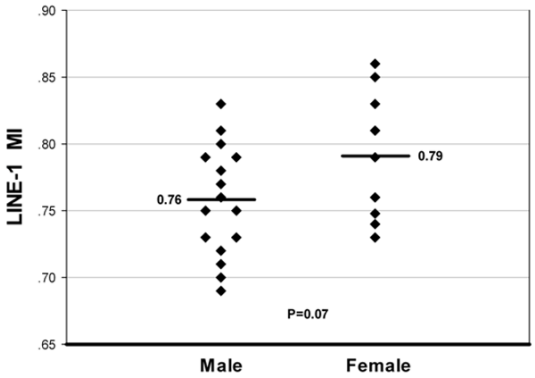

Figure 3. MI values of LINE-1 in males and females.

Discussion

To the best of our knowledge, LINE-1 methylation is a good indicator of the global DNA methylation level [15]. We estimated the methylation level of LINE-1 in APA and NA tissues using real-time methylation-specific PCR and found that LINE-1 hypomethylation is an important feature of APA. The MI of LINE-1 was 0.91 in NA tissue and 0.77 in APA tissue. There was a significant difference in the MI level of LINE-1 between APA and NA tissue, suggesting that LINE-1 hypomethylation is involved in APA formation as a molecular abnormality. Thus, LINE-1 hypomethylation has the potential to be a biomarker for APA.

According to our study, LINE-1 hypomethylation was not significantly associated with any clinicopathologic features. There was also no significant difference between males and females. Therefore, the associations between LINE-1 methylation and APA appears to be independent of gender, age, tumor diameter, maximum mean arterial pressure, basic plasma aldosterone concentration and aldosterone-renin ratio. Many studies [16, 17] have reported that gender is associated with LINE-1 methylation in blood DNA, with males having higher LINE-1 methylation levels. It is not known whether this gender difference is attributable to copy number variation in LINE-1 on the X- and Y-chromosomes or whether sex hormones influence methylation. As Kile et al. reported [18], maternal blood LINE-1 methylation is correlated with offspring LINE-1 methylation. This finding suggests the possibility that LINE-1 methylation levels may be genetically regulated. Thus, gender differences in LINE-1 methylation in blood DNA may be attributable to the sex chromosomes. However, based on our observations, females were tended to be higher LINE-1 methylation levels than males, although the difference was not significant. Additional studies will be required to confirm our results, due to the limits of sample size and tissue specificity in our study.

It is estimated that nearly half of the DNA content in the human genome is composed of repetitive sequences of DNA, such as transposons, retrotransposons and endogenous retroviruses. Typically these elements are non-transcribed and maintained as heterochromatin.

Thus, they are characterized as being hypermethylated. LINE-1 elements are the most abundant autonomous retrotransposons in the human genome [19]. A functional full-length LINE-1 element consists of a 5'UTR with an internal RNA polymerase II promoter, two open reading frames (ORF1,2) encoding an RNA binding protein and elements necessary for retrotransposon activity, and a 3'UTR containing a polyadenylation signal [9]. It has been suggested that the 5' end of the sequence tends to be deleted (but with an unknown frequency) except in more active, evolutionarily newer sequences, which are often present in somatic cells that have undergone malignant transformation [20].

Hypomethylation can cause LINE-1 elements to be transcribed. LINE-1 expression damages host DNA by causing insertions that disrupt gene expression [21] and acting as potent substrates for unequal homologous recombination that leads to gained or lost genomic sequences [22]. However, the detailed examination of LINE-1 methylation and its pathogenic mechanism has been limited by significant technical limitations because there are approximately 500,000 LINE-1 elements in the genome and it is not unknown how many of these are of full length. In our assay, we cannot know for sure how many elements we evaluate or whether this number is similar across samples or individuals [23]. It is thought that LINE-1 hypomethylation is associated with many disease states including many types of cancer [24-27], stroke [7] and heart disease [28]. However, there are few studies about LINE-1 methylation and tumors derived from the adrenal gland. Geli et al. [29] assessed LINE-1 methylation in pheochromocytomas and abdominal paragangliomas. Slightly lower levels of LINE-1 methylation were observed in the tumors compared with the normal adrenal samples ($P < 0.05$).

In our study, we observed obviously lower levels of methylated Line-1 in APA tissues. We hypothesize that the degree of LINE-1 methylation may be different in different tumors derived from the adrenal gland, but this hypothesis will require further studies to verify.

In summary, we report that LINE-1 methylation levels are significantly lower in APA tissue than in NA tissue, with no correlations between MI and clinical characteristics. Further studies using larger sample sizes and different tumor types derived from the adrenal gland should be carried out in the future.

Acknowledgements

This work was supported by the Shanghai Science and Technology Committee (grant numbers 09411954500).

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