

CHAPTER IV

RESULTS

Quantitated LINE-1 methylation status by COBRA LINE-1

To study genomic DNA methylation status of 5' UTR of LINE-1 by COBRA LINE-1, in this study used DNA samples from leukocytes, normal and cancer tissue from paraffin embedded sections and serum. Leukocytes from the peripheral blood of 16 women and 16 men were tested. In each group, half of subjects were younger than 40 years of age. Normal tissues and cancers in a broad panel of malignancies including urothelial carcinoma, head and neck squamous cell carcinoma, hepatocellular carcinoma, non-small cell lung carcinoma, renal cell carcinoma, prostatic adenocarcinoma, gastric adenocarcinoma, malignant lymphoma, papillary carcinoma of thyroid, mammary ductal carcinoma, colonic carcinoma, gastric cancers and esophageal squamous cell carcinoma were microdissected paraffin-embedded samples to isolate malignant cells from their adjacent normal epithelium or connective tissues. Serum was obtained from gastric cancer patients with similar age and sex match control. The DNA samples are treated with bisulfite which, unmethylated cytosine but not methylated will be modified to be uracil. After PCR, a specific restriction enzyme will be applied to differentiate methylated and unmethylated DNA. COBRA LINE-1 used *TaqI* cutting TCGA sequence, to digest amplicon from methylated DNA at nt 285 and *TasI* cutting AATT sequence for digesting amplicon from unmethylated DNA at nt 270. *TaqI* and *TasI* digests fragment 160 bp that, *TasI* gave amplicons 63 bp and 97 bp and *TaqI* 80 bp are two *TasI*. Quantitative analysis is performed with a molecular Dynamic phosphoimager. The percentage of hypomethylation can be calculated from measured intensity of *TasI* digestible amplicon divided by summed of *TasI* and *TaqI* product (figure 4-1). Figure 4- 2 represent example of COBRA LINE-1 in normal epithelium and esophageal cancer. The total result of quantitated LINE-1 methylation status indicated in table 4-1.

Table 4-1 Result of hypomethylation

MALE					FEMALE				
case	intensity of band (%)			%hypomethylation	case	intensity of band (%)			%hypomethylation
	TasI (97bp)	TasI (63bp)	TagI (80 bp)			TasI (97bp)	TasI (63bp)	TagI (80 bp)	
1	28.29	28.67	43.04	56.96	1	29.66	28.82	41.51	58.49
2	30.3	29.3	40.4	59.6	2	27.47	27.88	44.65	55.35
3	28.01	27.42	44.56	55.41	3	29.96	29.38	40.66	59.34
4	32.21	29.81	37.98	62.02	4	30	28.25	41.75	58.34
5	29.42	29.34	41.24	58.76	5	31.72	29.78	38.51	61.49
6	30.52	30.3	39.15	60.85	6	32.07	29.17	38.76	61.24
7	28.78	30.96	40.27	59.73	7	32.47	30.22	37.3	62.7
8	29.11	31.79	39.1	60.9	8	27.56	28.44	44	56
9	31.63	31.97	36.41	63.59	9	27.81	30.09	42.11	57.89
10	28.23	32.18	39.59	60.41	10	30.71	28.36	40.92	59.08
11	29.3	30.19	40.52	59.48	11	31.36	30.56	38.08	61.92
12	29.4	32.38	38.23	61.77	12	27	27.94	45.06	54.94
13	30.6	31.47	37.93	62.07	13	30.15	28.54	41.31	58.69
14	29.48	30.5	40.02	59.98	14	29.9	30.62	39.48	60.52
15	28.71	29.97	41.31	58.69	15	32.2	28.54	39.25	60.75
16	28.16	29.72	42.11	57.89	16	28.96	28.42	42.61	57.39

AGE<40					AGE>40				
case	intensity of band (%)			%hypomethylation	case	intensity of band (%)			%hypomethylation
	TasI (97bp)	TasI (63bp)	TagI (80 bp)			TasI (97bp)	TasI (63bp)	TagI (80 bp)	
1	31.63	31.97	36.41	63.59	1	28.29	28.67	43.04	56.96
2	28.23	32.18	39.59	60.41	2	30.3	29.3	40.4	59.6
3	29.3	30.19	40.52	59.48	3	28.01	27.42	44.56	55.41
4	29.4	32.38	38.23	61.77	4	32.21	29.81	37.98	62.02
5	30.6	31.47	37.93	62.07	5	29.42	29.34	41.24	58.76
6	29.48	30.5	40.02	59.98	6	30.52	30.3	39.15	60.85
7	28.71	29.97	41.31	58.69	7	28.78	30.96	40.27	59.73
8	28.16	29.72	42.11	57.89	8	29.11	31.79	39.1	60.9
9	27.81	30.09	42.11	57.89	9	31.63	31.97	36.41	63.59
10	30.71	28.36	40.92	59.08	10	28.23	32.18	39.59	60.41
11	31.36	30.56	38.08	61.92	11	29.3	30.19	40.52	59.48
12	27	27.94	45.06	54.94	12	29.4	32.38	38.23	61.77
13	30.15	28.54	41.31	58.69	13	30.6	31.47	37.93	62.07
14	29.9	30.62	39.48	60.52	14	29.48	30.5	40.02	59.98
15	32.2	28.54	39.25	60.75	15	28.71	29.97	41.31	58.69
16	28.96	28.42	42.61	57.39	16	28.16	29.72	42.11	57.89

URINARY BLADDER				
case	intensity of band (%)			%hypomethylation
	Tasl (97bp)	Tasl (63bp)	Tagl (80 bp)	
N1	34.44	31.27	34.29	65.71
N2	31.62	34.29	34.1	65.9
N3	34.83	30.87	34.3	65.7
N4	34.59	31.31	34.1	65.9
N5	33.19	31.17	35.64	64.36
N6	35.61	29.71	34.68	65.32
N7	34.26	31.61	34.13	65.87

UROTHELIAL CARCINOMA				
case	intensity of band (%)			%hypomethylation
	Tasl (97bp)	Tasl (63bp)	Tagl (80 bp)	
C1	34.54	34.38	31.09	68.91
C2	35.2	35.79	29.02	70.98
C3	35.13	33.53	31.35	68.65
C4	34.61	34.05	31.34	68.66
C5	33.83	31.53	34.63	65.37
C6	35.77	33.23	31	69
C7	37.3	32.83	29.87	70.13

HEAD AND NECK SQUAMOUS CELL				
case	intensity of band (%)			%hypomethylation
	Tasl (97bp)	Tasl (63bp)	Tagl (80 bp)	
N1	15.96	48.06	35.98	62
N2	14.84	43.61	41.54	58.46
N3	25.81	32.2	41.99	58.01
N4	32.33	32.92	34.75	65.25
N5	29.68	32.61	37.7	62.3
N6	26.67	32.71	41.16	58.84
N7	34.28	30.9	34.82	65.18
N8	33.91	30.45	35.64	64.36

HEAD AND NECK CARCINOMA				
case	intensity of band (%)			%hypomethylation
	Tasl (97bp)	Tasl (63bp)	Tagl (80 bp)	
C1	35.11	28.08	28.08	71.92
C2	16.87	33.62	33.62	66.38
C3	26.25	36.01	36.01	63.99
C4	33.83	32.02	32.02	67.98
C5	26.03	30.13	30.13	67.87
C6	33.05	34.58	34.58	65.42
C7	28.54	27.52	27.52	72.48
C8	28.95	32.47	32.47	67.53

LIVER				
case	intensity of band (%)			%hypomethylation
	Tasl (97bp)	Tasl (63bp)	Tagl (80 bp)	
N1	31.29	33.4	35.3	64.7
N2	32.22	32.98	34.8	65.2
N3	32.86	33.67	33.47	66.53
N4	32.81	31.81	35.38	64.62
N5	31.59	34.06	34.35	65.65
N6	31.29	33.4	35.3	64.7
N7	34.09	31.29	34.62	65.38
N8	31.31	33.39	35.31	64.69
N9	30.34	34.83	34.83	65.17

HEPATOCELLULAR CARCINOMA				
case	intensity of band (%)			%hypomethylation
	Tasl (97bp)	Tasl (63bp)	Tagl (80 bp)	
C1	36.11	34.95	28.94	71.06
C2	34.99	35.09	29.92	70.08
C3	35.21	35.05	29.73	70.27
C4	36.74	33.5	29.76	70.24
C5	36.32	36.62	27.06	72.94
C6	36.11	34.95	28.94	71.06
C7	34.74	34.3	30.96	69.04
C8	34.55	33.95	31.5	68.5
C9	32.56	35.07	32.37	67.63

LUNG				
case	intensity of band (%)			%hypomethylation
	TasI (97bp)	TasI (63bp)	TagI (80 bp)	
N1	28.08	32.84	39.07	60.93
N2	30.15	31.99	37.86	62.14
N3	31.05	32.39	36.56	63.44
N4	30.02	31.76	38.22	68.24
N5	29.92	31.52	38.56	61.44
N6	29.53	30.06	40.42	59.58
N7	30	32.22	37.79	62.21

NON-SMALL CELL LUNG CARCINOMA				
case	intensity of band (%)			%hypomethylation
	TasI (97bp)	TasI (63bp)	TagI (80 bp)	
C1	30.81	31.05	38.13	61.87
C2	33.08	33.5	33.41	66.59
C3	34.34	33.07	32.59	67.41
C4	31.07	34.5	31.56	68.44
C5	31.07	31.87	37.06	62.94
C6	33.41	33.71	32.88	67.12
C7	32.96	35.07	31.97	68.03

KIDNEY				
case	intensity of band (%)			%hypomethylation
	TasI (97bp)	TasI (63bp)	TagI (80 bp)	
N1	28.33	30.6	41.07	58.93
N2	29.88	29.72	40.4	59.6
N3	29.78	30.23	39.99	60.01
N4	30.5	28.41	41.1	58.9
N5	31.39	27.63	40.98	58.02
N6	29.56	29.7	40.75	59.25
N7	30.36	30.06	39.59	60.41
N8	30.18	29.43	40.39	59.61

RENAL CELL CARCINOMA				
case	intensity of band (%)			%hypomethylation
	TasI (97bp)	TasI (63bp)	TagI (80 bp)	
C1	29.91	28.38	41.7	58.3
C2	29.01	30.05	40.94	59.06
C3	30.45	31.39	38.15	61.49
C4	31.41	29.7	38.89	61.11
C5	28.62	29.76	41.62	58.38
C6	31.04	28.43	40.53	59.47
C7	30.65	29.38	39.97	60.03
C8	29.4	29.87	40.73	59.37

ศูนย์วิทยุทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

PROSTATE				
case	intensity of band (%)			%hypomethylation
	Tasl (97bp)	Tasl (63bp)	Tagl (80 bp)	
N1	30.51	30.1	39.39	60.61
N2	29.99	35.36	34.65	64.35
N3	29.41	35.14	35.45	62.45
N4	30.31	35.11	34.58	64.13
N5	33.87	33.3	32.83	67.17
N6	30.87	33.71	35.41	64.59
N7	38.31	37.65	24.04	75.96

PROSTATIC ADENOCARCINOMA				
case	intensity of band (%)			%hypomethylation
	Tasl (97bp)	Tasl (63bp)	Tagl (80 bp)	
C1	33.46	32.86	33.68	66.32
C2	29.55	34.8	35.65	65.35
C3	27.87	34.58	37.55	64.55
C4	30.46	33.67	35.87	65.42
C5	36.29	35.8	27.91	72.09
C6	30.97	34.63	34.4	65.6
C7	39.85	39.41	20.74	79.26

BREAST				
case	intensity of band (%)			%hypomethylation
	Tasl (97bp)	Tasl (63bp)	Tagl (80 bp)	
N1	30.5	32.31	37.19	62.81
N2	29.74	30.99	39.27	60.73
N3	28.61	31.71	36.69	60.31
N4	26.66	30.92	39.42	60.58
N5	28.4	31.13	40.47	59.53
N6	31.77	31.1	36.93	63.07
N7	29.14	31.17	39.68	60.32

MAMMARY DUCTAL CARCINOMA				
case	intensity of band (%)			%hypomethylation
	Tasl (97bp)	Tasl (63bp)	Tagl (80 bp)	
C1	32.07	33.55	34.38	65.62
C2	33.2	34.67	32.13	67.87
C3	30.68	33.62	35.7	64.3
C4	31.24	31.89	36.87	63.13
C5	29.82	31.4	38.79	61.21
C6	36.04	33.8	30.16	69.84
C7	30.09	34.37	35.54	64.46

ESOPHAGUS				
case	intensity of band (%)			%hypomethylation
	Tasl (97bp)	Tasl (63bp)	Tagl (80 bp)	
N1	22.02	35.85	42.13	57.87
N2	21.13	22.69	56.19	43.81
N3	16.04	18.38	65.58	34.42
N4	26.01	23.88	50.11	49.89
N5	31.76	32.56	35.68	64.32
N6	29.5	34.03	36.47	63.53
N7	28.01	40.49	31.51	68.49

ESOPHAGUAL SQUAMOUS CARCINOMA				
case	intensity of band (%)			%hypomethylation
	Tasl (97bp)	Tasl (63bp)	Tagl (80 bp)	
C1	38.05	26.62	35.33	64.67
C2	28.1	35.96	35.94	64.06
C3	26.24	23.85	49.9	50.1
C4	22.41	35.21	42.39	57.61
C5	33.81	38.65	27.54	72.46
C6	38.32	39.08	22.61	77.39
C7	32.79	37.33	29.88	70.12

THYROID				
case	intensity of band (%)			%hypomethylation
	TasI (97bp)	TasI (63bp)	TagI (80 bp)	
N1	33.3	40.41	26.29	73.71
N2	20.37	37.19	42.43	57.57
N3	30.67	24.64	44.69	55.31
N4	25.38	17.23	57.39	42.61
N5	23.86	26.97	49.17	50.83
N6	29.87	29.59	40.55	59.45
N7	20.22	24.83	54.95	45.05

PAPILLARY CARCINOMA OF THYROID				
case	intensity of band (%)			%hypomethylation
	TasI (97bp)	TasI (63bp)	TagI (80 bp)	
C1	28.26	45.8	25.94	74.06
C2	30.44	36.9	32.66	67.34
C3	24.67	31.5	43.84	56.16
C4	33.01	18.63	48.37	51.63
C5	24.38	26.16	49.46	50.54
C6	23.14	23.97	52.89	47.11
C7	24.58	24.8	50.62	49.38

STOMACH				
case	intensity of band (%)			%hypomethylation
	TasI(97bp)	TasI (63bp)	TagI (80 bp)	
N1	30.7	32.44	36.86	63.14
N2	31.28	33.25	35.46	64.81
N3	32.11	31.05	36.83	64.75
N4	33.23	30.1	36.67	63.71
N5	31.96	32.85	35.19	63.17
N6	30.87	32.03	37.1	63.33
N7	30.82	32.89	36.29	64.54
N8	32.56	32.19	35.25	62.9
N9	31.52	32.56	35.92	64.08

GASTRIC CARCINOMA				
case	intensity of band (%)			%hypomethylation
	TasI (97bp)	TasI (63bp)	TagI (80 bp)	
C1	34.8	35.6	29.6	70.4
C2	32.58	33	34.42	67.61
C3	32.46	33.01	34.53	66.53
C4	36.31	33.8	29.89	64.88
C5	33.41	34.21	32.39	65.47
C6	30.23	32.68	37.09	70.11
C7	32.33	32.55	35.12	65.58
C8	34.16	32.37	33.47	62.91
C9	33.58	31.68	34.74	66.53

LYMPH-NODE				
case	intensity of band (%)			%hypomethylation
	TasI (97bp)	TasI (63bp)	TagI (80 bp)	
N1	32.31	31.92	35.77	64.23
N2	30.91	31.36	37.73	62.27
N3	31.76	31.81	36.43	63.57
N4	30.38	31.77	37.85	62.15
N5	31.91	31.69	36.4	63.6
N6	31.5	30.7	37.8	62.2
N7	29.4	28.89	41.71	58.29

LYMPHOMAS				
case	intensity of band (%)			%hypomethylation
	TasI (97bp)	TasI (63bp)	TagI (80 bp)	
C1	31.55	30.93	37.52	62.48
C2	32.13	30.5	37.37	62.63
C3	33.42	31.08	35.49	64.51
C4	31.78	29.92	38.3	61.7
C5	32.5	29.55	37.95	62.05
C6	31.57	31.69	36.74	63.26
C7	30.32	30.19	39.49	60.51
C8	32.42	31.04	36.53	63.47

NORMAL EPITHELIUM OF COLON				
case	intensity of band (%)			%hypomethylation
	TasI (97bp)	TasI (63bp)	TagI (80 bp)	
N1	29.2	33.21	37.59	62.41
N2	31.1	33.75	35.15	64.85
N3	31.02	33.4	35.58	64.42
N4	31.08	33.05	35.86	64.13
N5	30.44	33.99	35.57	64.43
N6	31.25	33.2	35.55	64.45
N7	33.65	29.98	36.37	63.63
N8	34.59	28.92	36.48	63.52
N9	35.07	30.41	34.52	65.48
N10	30.49	34.4	35.11	64.89

COLONIC CARCINOMA				
case	intensity of band (%)			%hypomethylation
	TasI (97bp)	TasI (63bp)	TagI (80 bp)	
C1	32.77	37.18	30.06	69.94
C2	34.35	37.68	27.96	72.04
C3	31.31	33.14	35.55	64.45
C4	32.43	33.8	33.77	66.23
C5	31.38	33.71	34.91	65.09
C6	30.86	33.65	35.48	64.52
C7	34.91	31.11	33.98	66.02
C8	33.8	32.15	34.05	65.95
C9	35.39	33.12	31.49	66.88
C10	35.52	31.74	32.74	68.26

DYSPLATIC POLYPS				
case	intensity of band (%)			%hypomethylation
	TasI (97bp)	TasI (63bp)	TagI (80 bp)	
1	33.48	31.86	34.66	65.34
2	32.03	33.19	34.78	65.22
3	32.69	33.48	33.84	66.16
4	30.33	33.81	35.86	64.14
5	35.35	32.76	31.9	68.1
6	32.83	33.26	33.9	66.1
7	32.54	32.06	35.4	64.6
8	34.98	29.07	35.95	64.05
79	33.9	29.37	36.73	63.27
10	32.95	33.41	33.63	66.37
11	28.74	34.05	37.21	62.79
12	29.47	35.55	34.98	65.02
13	30.71	33.87	35.42	64.58
14	33.22	32.31	34.47	65.26
15	29.49	33.18	37.32	62.68
16	27.71	36.55	35.74	64.26

MUSCLE OF COLON				
case	intensity of band (%)			%hypomethylation
	TasI (97bp)	TasI (63bp)	TagI (80 bp)	
1	29.23	33.83	36.94	63.06
2	32.21	34.07	33.72	66.28
3	32.43	33.8	33.77	66.23
4	31.85	33.64	34.51	65.49
5	32.08	30.03	34.89	65.11
6	35.89	30.9	33.21	66.79

CONNECTIVE TISSUE OF COLON				
case	intensity of band (%)			%hypomethylation
	TasI (97bp)	TasI (63bp)	TagI (80 bp)	
1	29.62	34.34	36.05	63.95
2	31.62	33.48	34.9	65.1
3	32.2	33.59	34.21	65.79
4	32.2	33.59	34.21	65.79
5	31.47	32.66	35.87	64.13
6	33.23	34.02	32.75	67.25

NORMAL SERUM				
case	intensity of band (%)			%hypomethylation
	TasI (97bp)	TasI (63bp)	TagI (80 bp)	
N1	29.48	30.07	40.45	59.55
N2	29.93	28.2	41.87	58.13
N3	25.43	27.78	46.8	53.2
N4	32.5	30.48	37.03	62.97
N5	29.96	28.1	41.94	58.06
N6	33.04	32.33	34.63	65.37
N7	29.64	29.44	40.92	59.08
N8	31.09	27.16	41.75	58.25
N9	33.2	31.04	35.76	64.24
N10	31.79	31.89	36.32	63.68
N11	25.54	29.26	45.2	54.8
N12	33.18	33.61	33.21	66.79
N13	29.81	31.82	38.37	61.63
N14	28.08	28.74	43.17	56.83
N15	28.3	28.18	43.52	56.48
N16	27.88	30.32	41.81	58.19
N17	29.08	31.05	39.87	60.13

SERUM OF GASTRIC CANCER				
case	intensity of band (%)			%hypomethylation
	TasI (97bp)	TasI (63bp)	TagI (80 bp)	
C1	27.51	33.37	39.12	60.88
C2	28.17	28.42	43.41	56.59
C3	34.73	32.97	32.29	67.71
C4	31.81	30.26	37.93	62.07
C5	31.11	27.91	40.98	59.02
C6	32.27	28.11	39.62	60.38
C7	30.53	26.27	43.21	56.79
C8	32.83	32.99	34.18	65.82
C9	28.09	43.53	28.38	71.62
C10	26.34	46.24	27.41	72.59
C11	27.59	41.96	30.45	69.55
C12	24.94	47.37	27.69	72.31
C13	27.71	40.19	32.1	67.9
C14	28.34	29.27	42.39	57.61
C15	27.6	28.64	43.76	56.24
C16	31.49	30.34	38.17	61.83
C17	29.07	43.72	27.21	56.28

จุฬาลงกรณ์มหาวิทยาลัย

Data analysis

1. Evaluated the differential level genomic hypomethylation of LINE -1 in different gender and age.

The experiment studied correlation of genomic LINE-1 hypomethylation in different gender and age. This experiment analyzed normal leukocytes from peripheral blood by comparing between male and female and between age<40 and age>40 (n=16). The hypothesis is that women and youth have more methylated DNA than the men and the elderly people because X-inactivation and increased hypomethylation in aging respectively. The data was analyzed by independent sample T-test ($H_0: \mu_1 > \mu_2$, $H_1: \mu_1 = \mu_2$).

From table 4-2 and figure 4-3 A show no difference level of LINE-1 hypomeyhylation between male and female ($p > 0.1$) and between elderly and youth ($p > 0.5$).

cases	T-test for Equality of Means						
	t	df	Sig. (1-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval	
						Lower	Upper
male - female	1.117	30	0.137	0.873	0.781	-0.723	2.470
age>40-age<40	-0.621	30	0.270	-0.492	0.792	-2.111	1.126

2. Level of genomic hypomethylation of LINE-1 in difference normal tissue.

Differences of hypomethylation among epithelium tissue, connective tissue and muscle in the same organ was studied in 7 match cases of normal colons by paired sample T-test. The hypothesis is that mean of hypomethylation different among tissues ($H_0: \mu_1 \neq \mu_2$, $H_1: \mu_1 = \mu_2$). From table 4-3 show significant greater hypomethylation of connective tissues ($p < 0.05$) and muscle ($p < 0.01$) were detected than those of normal epithelium. Next we sought to characterize the levels of genomic hypomethylation among normal tissues including urinary bladder, head and neck, liver, lung, prostate gland, stomach, colon, breast, kidney and esophagus analyzed by independent sample T-test ($H_0: \mu_1 \neq \mu_2$, $H_1: \mu_1 = \mu_2$). From table 4-6 show significant difference level compared between normal tissues types. For example, the levels of LINE-1 hypomethylation of urothelium ($p < 10^{-10}$), gastric epithelium ($p < 10^{-9}$), colon epithelium ($p < 10^{-10}$) and liver epithelium ($p < 10^{-11}$) were higher than of renal epithelium. In most cases, the distribution of detectable LINE-1 hypomethylation within individual normal tissue was consistently cluster with in 5% range, whereas the hypomethylaton in thyroid (42.61-73.71%) and esophageal tissue (34.42-68.49%) were widely distributed (table 4-4).

Table 4-3 Paired sample T-test for compared hypomethylation of difference colon tissues

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviat ion	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	connective - epithelium	1.22	1.101	0.4497	0.0641	2.3759	2.713	5	0.021
Pair 2	muscle - epithelium	1.3783	0.653	0.2666	0.693	2.0637	5.17	5	2.00E-03

Table 4-4 Percentage of hypomethylation in normal tissues

type of normal tissues	%hypomethylation	mean
colon	62.41 - 65.48	64.22
bladder	64.36 - 65.90	65.54
head&neck	58.01 - 65.25	61.85
liver	64.62 - 66.53	65.18
lung	59.58 - 68.24	62.57
kidney	58.02 - 60.41	59.34
prostate	60.61 - 75.96	65.61
breast	59.53 - 63.07	61.05
esophagus	34.42 - 68.49	54.62
thyroid	42.61 - 73.71	54.93
lymph-node	58.29 - 64.23	62.33
stomach	62.90 - 64.81	63.83

Table 4-5 Percentage hypomethylation in cancer tissues

type of cancer tissues	%hypomethylation	mean
colon	64.45 - 72.04	66.94
bladder	65.37 - 70.98	68.81
head&neck	63.99 - 72.48	67.95
liver	67.63 - 72.94	70.09
lung	61.87 - 68.44	66.06
kidney	58.30 - 61.49	59.65
prostate	64.55 - 79.26	68.37
breast	61.21 - 69.84	65.20
esophagus	50.10 - 77.39	65.20
thyroid	47.11 - 74.06	56.60
lymph-node	60.51 - 64.51	62.58
stomach	62.91 - 70.40	66.67

Table 4-6 Significant value (sig.(2tailed)) of independent sample T-test for compared between normal tissues

	colon	bladder	head&neck	liver	lung	kidney	prostate	breast	esophagus	thyroid	lymph node	stomach
colon		3.00E-03		1.30E-03		9.27E-10		2.70E-05			1.00E-02	
bladder	3.00E-03		1.10E-03		1.70E-03	1.36E-10		3.14E-06		3.50E-03	1.00E-03	1.70E-04
head&neck		1.10E-03		1.70E-03								
liver	1.30E-03		1.70E-03		4.70E-03	1.89E-11		1.02E-06		4.00E-02	1.00E-03	1.00E-03
lung		1.70E-03		4.70E-03		7.00E-03						
kidney	9.27E-10	1.36E-10		1.89E-11	7.00E-03		1.60E-03	8.00E-03			1.00E-03	2.65E-09
prostate						1.60E-03		3.80E-03		3.00E-02		
breast	2.70E-05	3.14E-06		1.02E-06		8.00E-03	3.80E-03					1.19E-04
esophagus												
thyroid		3.50E-03		4.00E-02			3.00E-02					
lymph node	1.00E-02	1.00E-03		1.00E-03		1.00E-03						
stomach		1.70E-04		1.00E-03		2.65E-09		1.19E-04				

3. Evaluated the differential level genomic hypomethylation of LINE-1 between normal and cancer tissues.

In this experiment evaluated the differential level of LINE-1 methylation between normal tissues and cancers in a broad panel of malignancies including urothelial carcinoma, head and neck squamous cell carcinoma, hepatocellular carcinoma, non-small cell lung carcinoma, renal cell carcinoma, prostatic adenocarcinoma, gastric adenocarcinoma, malignant lymphoma, papillary carcinoma of thyroid, mammary ductal carcinoma, and esophageal squamous cell carcinoma. For this purpose, we manually microdissected paraffin-embedded samples to isolate malignant cells from their adjacent normal epithelium or connective tissues. Hypothesis is that malignancies more hypomethylated than normal epithelium ($H_0: \mu_1 < \mu_2$, $H_1: \mu_1 = \mu_2$). Match case analysis was documented by pair sample T-test. Lymphomas were compared with reactive lymph nodes of the sex- and age-matched different individuals analyzed by independent sample T-test. Comparing to their normal tissue counterparts, significantly greater hypomethylation was demonstrated in most cancers including carcinomas of

urinary bladder, head and neck, liver, lung, prostate gland, stomach, colon, breast, and esophagus($p < 0.05$). And, no difference level of LINE-1 hypomethylation between normal and cancer in kidney thyroid and lymph-nodes ($p > 0.05$)(Table 4-7, Figure 4-3 B).

The distribution of global hypomethylation level of each tumor type corresponded well to the different cellular differentiation. For example, there were wide ranges of COBRA LINE-1 hypomethylation within esophageal carcinoma (50.10-77.39%) and their normal counterparts (34.42-68.49%) whereas those of urothelial carcinomas (65.37-70.98%) in cancer and 64.36-65.90% in normal tissue were relatively clustered (table4-5,table4-6).

From figure 4-4 The mean of an increased level of hypomethylation ranged from 3-6% in most cancers, but varied from 2.82% in prostate and 10.58% in esophageal carcinomas (fig. 4-3). It is interesting to note that there were possible two groups of differential genomic hypomethylation in cancers, low (0-3.4%) or moderately high group (6.8-9.5), as could be demonstrated in gastric, head and neck, and colonic carcinoma .



ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Table 4-7 T-test for compared hypomethylation between normal and cancer tissues

	Paired Differences					t	df	Sig. (1-tailed)
	Mean diff	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
normal bladder - urothelial carcinoma	-3.28	1.28	0.49	-4.47	-2.09	-6.75	6	5.00E-04
squamous cell head&neck - head&neck carcinoma	-6.10	2.29	0.81	-8.01	-4.18	-7.51	7	5.00E-05
normal liver - hepatoma	-4.91	1.60	0.53	-6.14	-3.68	-9.20	8	1.00E-05
normal lung - lung carcinoma	-3.49	2.72	1.03	-6.00	-0.98	-3.40	6	7.50E-03
normal kidney - renal cell carcinoma	-0.31	1.03	0.36	-1.17	0.55	-0.85	7	0.2105
normal prostate - prostatic adenocarcinoma	-2.76	1.93	0.73	-4.55	-0.97	-3.78	6	4.50E-03
normal breast - mammary ductal carcinoma	-4.15	2.09	0.79	-6.09	-2.22	-5.25	6	1.00E-03
normal esophagus - esophageal carcinoma	-10.58	6.31	2.38	-16.42	-4.75	-4.44	6	2.00E-03
thyroid - thyroid carcinoma	-1.67	7.41	2.80	-8.52	5.18	-0.60	6	0.2865
normal stomach - gastric carcinoma	-2.84	2.52	0.84	-4.78	-0.91	-3.39	8	5.00E-03
normal lymphnodes-lymphoma	-0.25	1.6	0.83	-2.04	1.55	-0.30	13	3.86E-01

4. Evaluated level genomic hypomethylation of LINE-1 in the multistep carcinogenesis.

Colonic tissues were selected as a model to address roles of LINE-1 hypomethylation in the multistep carcinogenesis. Dysplastic polyps analyzed by independent sample T-test. From previous study which, decrease of methylation was found early event in carcinogenesis. Therefore, Hypothesis are polyp more hypomethylation than normal and carcinoma more hypomethylation than polyp and normal. This result found LINE-1 hypomethylation in the late stage of malignant progression in colonic carcinoma as could be seen by significantly more hypomethylation in the cancer comparing to dysplastic polyps ($p < 0.01$) and normal epithelium ($p < 0.01$). But, adenomatous polyps appeared to be more hypomethylated than normal epithelium, the difference was not statistically significance ($p = 0.102$) (table 4-8, figure 4-3C).

Table 4-8 T-test for compared multistep in colonic carcinoma

cases	t-test for Equality of Means						
	t	df	Sig. (1-tailed)	Mean Difference	Std. Error Difference	95% Confidence	
						Lower	Upper
normal colon-polyps	-1.31	24	0.102	-0.65	0.497	-1.676	0.375
colonic carcinoma - polyps	2.73	24	5.50E-03	2.07	0.755	0.506	3.626
normal colon-colonic carcinoma	-3.8	10	2.00E-03	-2.96	0.79	-4.717	-1.206

5. Evaluated the differential level genomic hypomethylation of LINE-1 between serum gastric cancer and controls serum.

Finally, studied possibility of using COBRA LINE-1 as a potential tumor marker. Since circulating DNA in plasma or serum has recently become a promising tumor marker. we evaluated. By comparing between circulating DNA derived from serum of 17 gastric carcinoma patients together with 17 age- and sex-matched controls. Serum from the cancer patients consistently demonstrated greater hypomethylation levels (56.24-72.59%) as compared to the matched controls (53.20-66.79%), with a statistical significance ($p < 0.05$) (table4-3C, figure4-3D).

Table 4-9 Independent Samples T- Test for compared serum gastric cancer patient

cases	t-test for Equality of Means						
	t	df	Sig. (1-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
						Lower	Upper
normal serum - gastric cancer	-1.98	26.92	0.0285	-3.4	1.721	-6.932	0.132

ศูนย์วิทยุทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

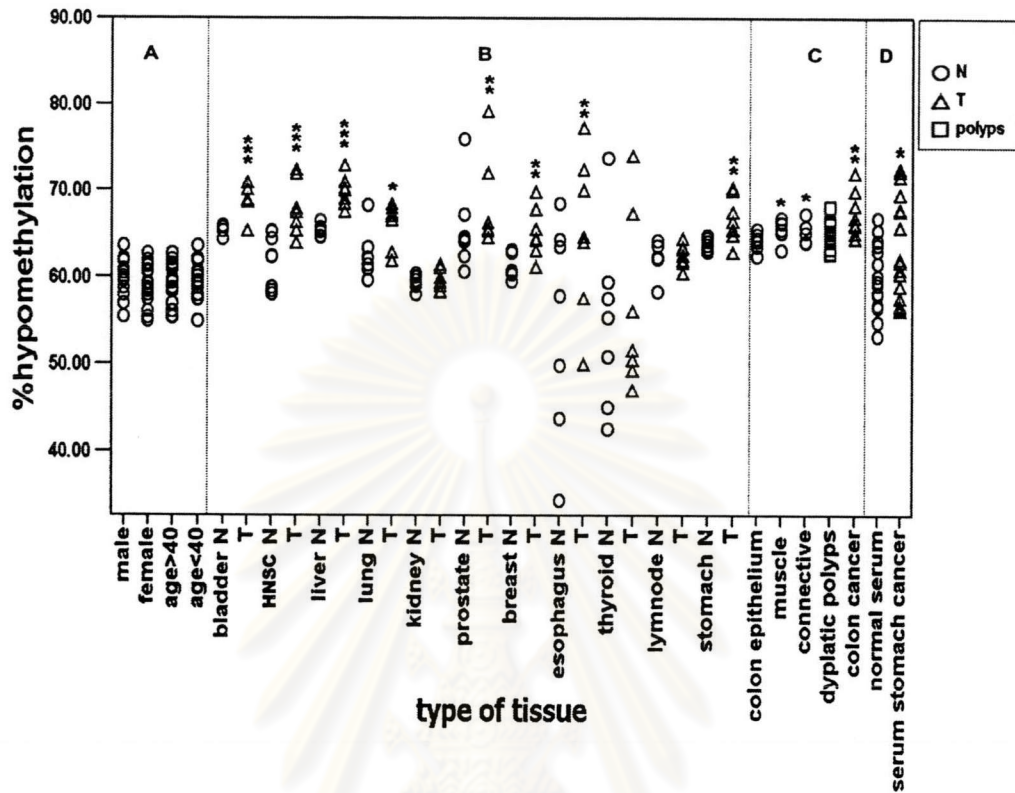


Figure 4-3. LINE-1 hypomethylation level of several tissue types. Circle, triangle and square are the level of each normal, malignant and premalignant tissues, respectively. The location regarding vertical axis is percentage level of LINE-1 methylation. Sample types are labeled. A, B, C, and D are the level of leukocytes, cancers, microdissected tissue colonic tissues and normal counterparts, and sera, respectively. One, two, and three asterisks indicate significances of difference of hypomethylation levels between normal tissues and the tested samples at $p < 0.05$, < 0.01 , and < 0.001 , respectively. HNSC is head and neck squamous cell. N and T are normal and malignant tissues, respectively.

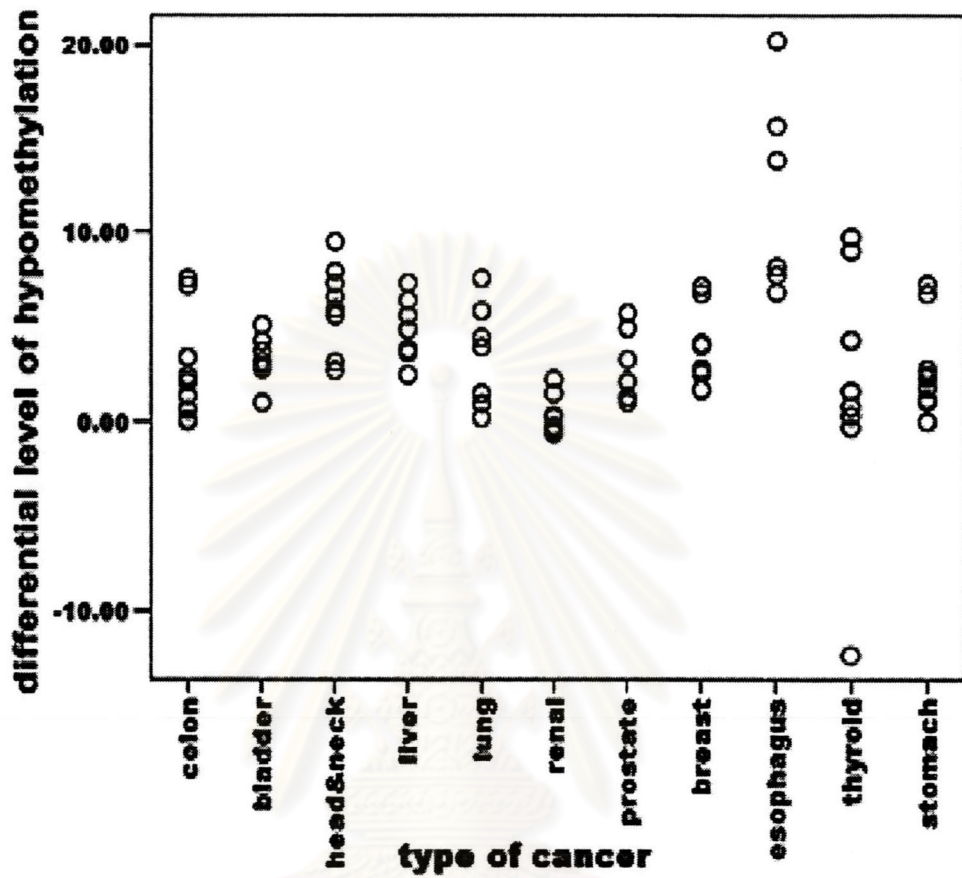


Figure 4-4. Differential level of LINE-1 hypomethylation in cancers from their adjacent normal tissues. The location of each circle in vertical axis shows LINE-1 hypomethylation level of each cancer subtracted by hypomethylation level of its normal counterpart. Type of each cancer is listed under the samples.

Table 4-10 Summary of LINE-1 hypomethylation level in several tissue types

		%hypomet	mean			%hypomet	mean	<i>p</i>	95% CI	
									lower	upper
A. Leukocyte										
Female		54.94 - 62.70	59.01	Male		55.41 - 63.59	59.88	0.137	-0.72	2.47
<40 yrs		54.94 - 63.59	59.69	>40 yrs		55.35 - 62.70	59.20	0.27	-2.11	1.13
B. Colon										
Normal		62.41 - 65.48	64.22	CNT		63.95 - 67.25	65.33	0.02 ^a	0.06	2.38
Epithelium				Muscle		63.06 - 66.79	65.49	0.002 ^a	0.69	2.06
				Polyp		62.68 - 68.10	64.87	0.102 ^b	-1.68	0.37
				Cancer		64.45 - 72.04	66.94	0.0055	-4.63	-0.8
C. Normal versus Cancer										
Normal			Cancer							
Bladder		64.36 - 65.90	65.54			65.37 - 70.98	68.81	0.0005	-4.46	-2.09
Head & neck		58.01 - 65.25	61.85			63.99 - 72.48	67.95	0.00005	-8.01	-4.18
Liver		64.62 - 66.53	65.18			67.63 - 72.94	70.09	0.00001	-6.14	-3.68
Lung		59.58 - 68.24	62.57			61.87 - 68.44	66.06	0.0075	-6.00	-0.98
Kidney		58.02 - 60.41	59.34			58.30 - 61.49	59.65	0.211	-1.17	0.55
Prostate		60.61 - 75.96	65.61			64.55 - 79.26	68.37	0.0045	-4.55	-0.97
Breast		59.53 - 63.07	61.05			61.21 - 69.84	65.20	0.001	-6.09	-2.22
Esophagus		34.42 - 68.49	54.62			50.10 - 77.39	65.20	0.002	-16.42	-4.75
Thyroid		42.61 - 73.71	54.93			47.11 - 74.06	56.60	0.287	-8.52	5.18
Lymph node		58.29 - 64.23	62.33			60.51 - 64.51	62.58	0.385 ^b	-2.04	1.55
Stomach		62.90 - 64.81	63.83			62.91 - 70.40	66.67	0.005	-4.78	-0.91
D. Serum of stomach cancer patients										
Control		53.20 - 66.79	59.85	Patient		56.24 - 72.59	63.25	0.0285 ^b	-6.93	0.13

A, B, C, and D were comparison of genomic hypomethylation between normal peripheral blood leukocytes of different sex or age, microdissected tissue types of colon, cancer and normal counterpart, sera of stomach cancer patients and matched sex and age control individuals, respectively. %hypomet, percentage level of LINE-1 hypomethylation; *p*, *p* value; 95%CI, 95% confidence interval of the difference; < and > 40 yrs, age below or above 40 years old, respectively; CNT, connective tissue; Polyp, dysplastic polyp; *p* value and 95%CI were calculated by pair t-test one tailed analysis of most cases besides a and b. a, *p* value and 95%CI calculated by pair t-test two tailed analysis; b, *p* value and 95%CI calculated by unpair t-test one tailed analysis.