CHAPTER IV

RESULTS

CU-L1 and COBRA L1

To evaluate the level of methylation of each L1 locus and compare with COBRA-L1, we established a new technique COBRA 5' unique sequence to L1 (CU-L1) using similar strategy as COBRA-L1 (Fig. 1). In COBRA-L1, we converted DNA by bisulfite so that C will be U and T after amplification. However, methyl C will not be changed. Therefore, TasI and TaqI sequences will be created at unmethylated and methylated L1 respectively. After applying both restriction enzyme, unmethylated and methylated L1s will yield, 98 and 80 bp DNA, respectively. For CU-L1, we replaced bisulfited 5'L1 oligo with bisulfited unique sequence located 5' to L1 (Fig. 4.1). Interestingly, several CU-L1s showed more distinctive degrees of loss of methylation status during cancer progression (Fig. 4.1). In this study, CU-L1 and COBRA-L1 were performed to analyze several HNSCC cell lines, Leukemic cell lines, an Epithelial cell line, HNSCC microdissected specimens, Normal oral epithelial and Normal white blood cell samples. Figure 4.1 compare the strategies of COBRA-L1 and CU-L1. Each CU-L1 loci means the specific L1 methylation status within genome while COBRA-L1 showed L1's methylation of all L1 in genome. Both techniques were applied to measure hypomethylation level from the ratio of unmethylated band (98 bp) per summation of unmethylated band and methylated band density (98 bp + 80 bp). In figure 4.1 each lane show hypomethylation level of sample from HNSCC cell lines (HN), HNSCC microdissected specimen (M) and Normal oral epithelial (Or).

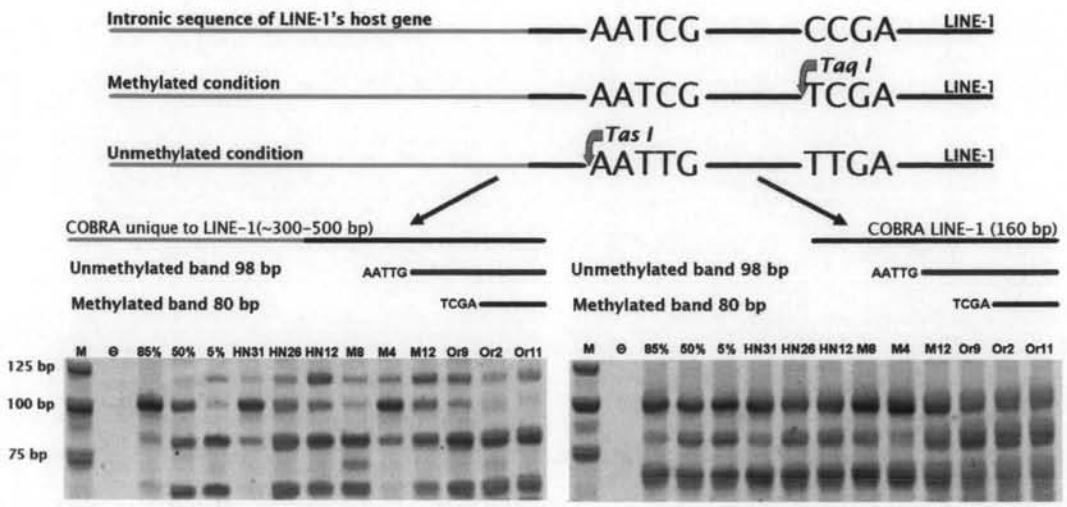

Figure 4-1: The schematic representation and examples of CU-L1 (left) and COBRA L1 (right).

CU-L1 PCR sequencing

To evaluate how CU-L1 level represent DNA methylation pattern of the L1 loci, CU-L1 amplicons of two loci were cloned and sequences. Black circle represent methylated CpG and white circle mean unmethylated CpG. PPP2R2B CU-L1, figure 4.2, in clone 4 show unmethylated condition. ANTXR2 CU-L1 sequence were show in figure 4.3 and 4.4. All type of methylation were sequencing, methylation type are mostly methylated in Taq I site cut. Unmethylated type usually loss methylation at Tas I site cut or completely loss methyl group from CpG loci in CU-L1 region. The result indicated that hypermethylated and hypomethylated CU-L1s generally represented, complete and no methylation of L1s on the loci. However, when CU-L1 methylation level was intermediate such as 50%, the methylation pattern was not heterozygote but partial methylation of each allele.

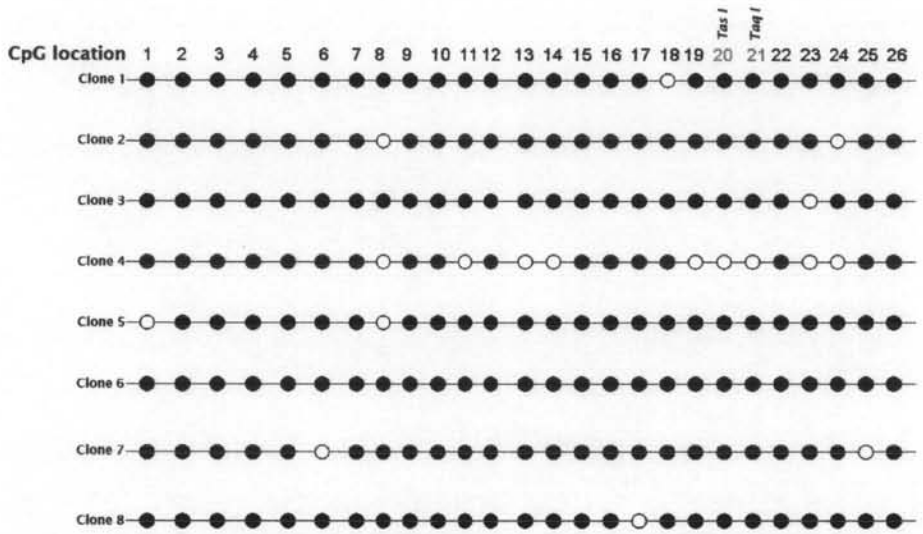

Figure 4.2 : Sequencing result of PPP2R2B CU-L1 from normal oral epithelial ; 58.08 % hypomethylation (Partial-met type)

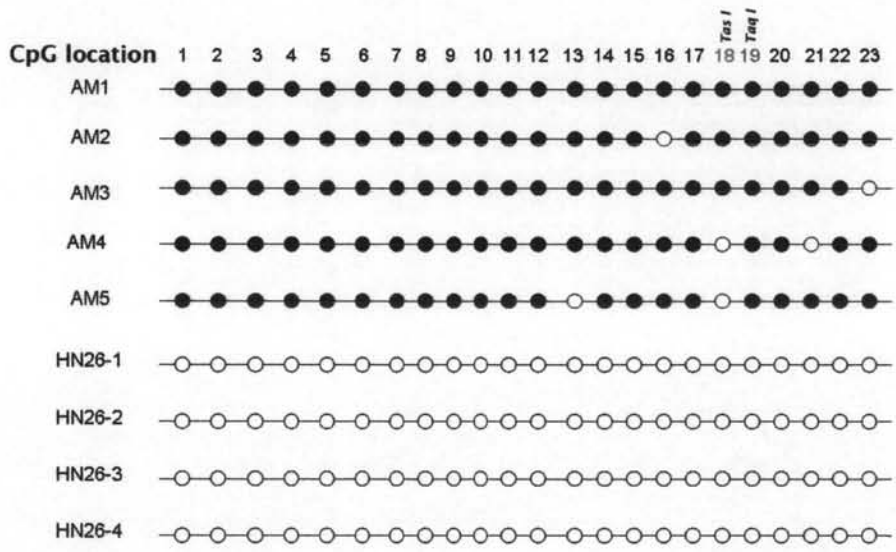

Figure 4.3 : Sequencing result of ANTXR2 CU-L1 from normal oral epithelial(AM) ; 2.89% Hypomethylation (Hypermet type) and from HNSCC cell line(HN26) ; 97.5 % Hypomethylation (Hypomet type).

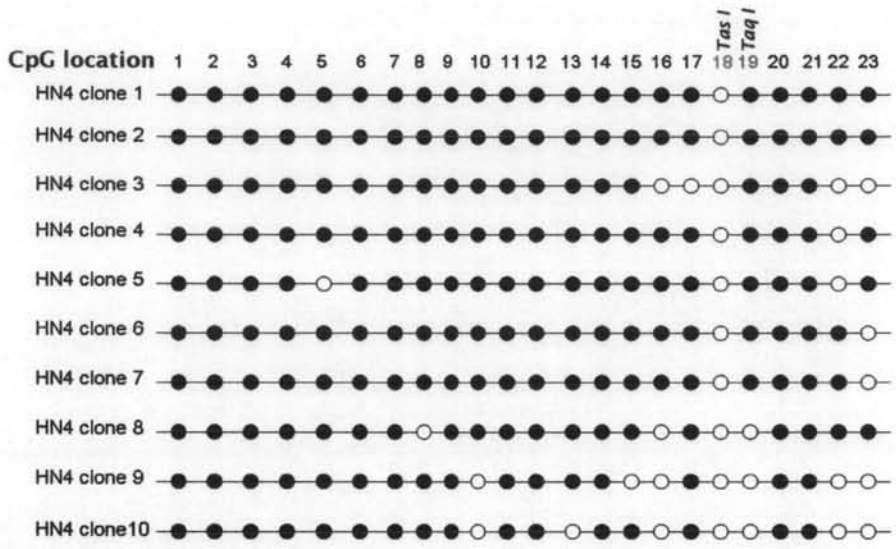

Figure 4.4 : Sequencing result of ANTXR2 CU-L1 from HNSCC cell line (HN4) ; 46.06 % Hypomethylation (partial-met type).

CU-L1 level represent carcinogenesis

Pearson correlation between hypomethylation from COBRA-L1 and mean of hypomethylation by CU-L1 is 0.913 while sig. (2-tailed) is less than 0.01. The linear correlation between hypomethylation level by COBRA-L1 and CU-L1 from all sample group was shown by graph in figure 4.5.

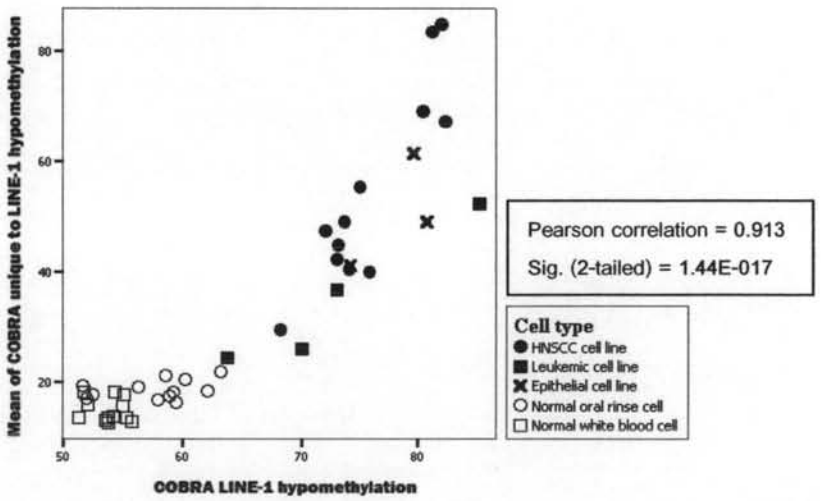

Figure 4.5 : Scatter plot show correlation between COBRA L1 and Mean of COBRA unique to L1.

Table 4.1 shown each study group in perform pearson correlation between COBRA-L1 hypomethylation and mean of hypomethylaion by CU-L1. While COBRA-L1 can represent carcinogenesis and normal tissue differentiation, CU-L1 can represent just carcinogenesis in HNSCC and Leukemic (pearson correlation is 0.904 and 0.968 with significant less than 0.01 and 0.05 respectively). This indicated that while genome wide evaluation did, the variation of L1 methylation within each normal tissue were not observed from these 17 L1 loci. In contrast, since COBRA-L1 represents global hypomethylation during cancer development, the loss of CU-L1 methylation level should be related to carcinogenesis.

Table 4.1: Hypomethylation correlation between COBRA L1 and COBRA unique to L1 of each specimen group.

Sample group	Pearson Correlation	Sig(2-tailed)	Number
HNSCC cell line	0.904	0.00005	12
Leukemic cell line	0.968	0.032	4
Epithelial cell line	0.701	0.506	3
Normal oral rinse	0.365	0.244	12
Normal WBC	-0.141	0.662	12

<u>Variation of L1 hypomethylation among loci and evidence of selective clonal expansion</u>

Hypomethylation level from CU-L1 of each sample group are report in Table 4.2 (HNSCC cell line), Table 4.3 (Leukaemic cell line), Table 4.4 (Epithelial cell line), Table 4.5 (HNSCC microdissected specimen), Table 4.6 (Normal oral epithelial) and Table 4.7 (Normal white blood cell). Figure 4.6 demonstrated levels of COBRA-L1 and CU-L1 loci from each cell type. CU-L1 methylation level from each loci are different. For example, PRKG1 have high level of hypomethylation (PRKG1 CU-L1 mean is not less than 50%) while PKP4 is low (PKP4 CU-L1 mean is not higher than 20%). Table 4.8 display ANOVA analysis in significant value for confirm potential of each L1 hypomethylated loci in examined cancer progression. Box plot in figure 4.6 exhibited loss of methylation level during carcinogenesis in both *in vitro* by compare HNSCC cell line with Normal oral

epithelial, and *in vivo*, comparing HNSCC microdissected with Normal oral epithelial. In Table 4.8, 9 CU-L1 loci indicated cancer progression both *in vitro* and *in vivo* including FAM49A, LOC286094, LRP2, CDH8, CNTNAP5, LOC133993, PPP2R2B, PKP4 and PRKG1. 2 CU-L1 loci separated only *in vitro* level, ADAMTS20 and LOC284395. 4 CU-L1 loci distinguish only *in vivo* level include ANTXR2, COL24A1, EPHA3 intron 5 and EPHA3 intron 15. More detail of multiple comparison by ANOVA analysis as seen in table 4.8 were in appendix D.

Table 4.2 : Hypomethylation of HNSCC cell line.

COBRA	Hypomethylation of Head and neck squamous cell carcinoma											
	HN4	HN6	HN8	HN12	HN13	HN17	HN19	HN22	HN26	HN30	HN31	KB
PKP4	1.2	0.73	2.04	1.08	1.04	1.4	1.98	1.43	1.47	1.54	0.65	28.58
EPHA3 intron 15	6.4	53.29	31.14	2.65	47.23	9.2	19.29	2.27	34.92	65.6	95.43	30.99
ANTXR2	46.04	95.26	12.44	9.99	12.12	14.26	95.48	10.95	97.5	93.87	80.91	76.83
EPHA3 intron 5	3.56	*	35.3	8.25	51.43	4.88	16.72	5.79	36.64	83.94	96.11	36.36
COL24A1	26.71	93.04	40.38	38.84	33.2	18.24	98.27	48.25	22.28	90.5	93.55	8.54
ADMTS20	10.78	74.56	43.6	22.77	27.67	10.26	97.76	12.28	26.72	94.52	95.66	53.49
SPOCK3	0	0	38.83	6.78	35.97	27.19	0.72	0	0	57.21	55.32	39.43
MGC42174	2.54	7.27	3.27	0	0	2.03	34.22	5.09	82.91	99.24	96.74	9.6
LOC284395	10.24	100	75.57	*	57.74	*	95.84	*	26.14	93.22	98.46	29.89
FAM49A	78.27	95.48	41.33	53.09	41.49	22.76	97.31	55.37	98.18	99.55	100	59.05
LOC286094	91.37	100	65.25	70.74	61.87	45.67	98.93	82.75	73.16	100	100	78.25
LRP2	60.14	73.91	100	69.49	51.84	74.59	98.78	69.82	29.21	100	100	68.15
CDH8	100	97.19	74.55	100	96.84	53.17	98.73	98.71	78.79	97.81	97.33	54.68
CNTNAP5	68.81	80.88	2.24	100	94.3	68.16	100	99.13	37.99	94.78	99.28	74.83
LOC133993	97.02	94.68	73.5	90.85	84.4	48.98	87.49	73.79	75.12	90.73	90.03	58.35
PPP2R2B	65.85	90.75	90.84	60.16	57.93	59	72.33	59.82	100	97.16	98.92	72.31
PRKG1	98.89	96.62	94.92	96.8	96.15	93.33	95.34	98.98	97.21	97.15	98.36	92.18
COBRA L1	73.01	82.35	71.97	74.09	72.94	68.15	80.45	75.97	75.07	81.22	82.01	73.59

Table 4.3 : COBRA unique to L1 hypomethylation of leukaemic cell line.

	Hypomethylation of leukaemic cell line			
COBRA	Daudi	Jurkat	Molt4	K562
PKP4	0.86	0.81	3.92	8.09
EPHA3 intron 15	46.14	12.47	33.3	96.76
ANTXR2	10.32	18.32	3.61	1.72
EPHA3 intron 5	13.61	11.85	4.16	94.51
COL24A1	12.23	18.07	14.51	92
ADMTS20	15.25	28.26	26.59	7.83
SPOCK3	32.24	0	46.05	9.38
MGC42174	4.76	28.45	18.45	9.62
LOC284395	8.15	61.53	10.82	92.7
FAM49A	14.66	16.37	47.12	90.04
LOC286094	4.3	6.62	18.57	94.21
LRP2	8.1	7.37	62.15	48.79
CDH8	66.9	78.05	62.09	76.08
CNTNAP5	0.9	18.11	44.13	99.35
LOC133993	44.5	48.64	69.73	64.87
PPP2R2B	58.11	76.62	73.9	81.63
PRKG1	92.62	67.66	91.8	91.92
COBRA L1	63.75	69.98	72.94	85.1

Table 4.4 : COBRA unique to L1 hypomethylation of Epithelial cancer cell line.

type	Hela	BC	SW480
PKP4	10.72	0	0
epha3(15)	47.51	0	72.01
Antxr2	30.83	100	45.91
epha3(5)	0	0	35.47
COL24A1	61.46	32.67	13.8
ADMTS20	85.22	85.17	12.63
SPOCK3	73.09	7.64	16.27
MGC42174	7.23	9.06	12.82
LOC284395		20.63	6.85
FAM49A	95.14	9.12	39.91
LOC286094	94.61	60.54	85.22
LRP2	79	22.64	78.75
CDH8	76.95	54.29	100
CNTNAP5	100	60.38	72.41
LOC133993	64.86	58.56	88.2
PPP2R2B	73.75	97.94	74.37
PRKG	91.28	82.97	91.33
COBRAL1	79.71	74.1	80.73

Table 4.5 : COBRA unique to L1 hypomethylation of HNSCC micordissect specimen.

	Hypomethylation of HNSCC microdissected specimen																	
COBRA	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18
PKP4	28.67	11.52	78.75	13.3	6.2	8.46	8.34	4.1	39.36	5.65	36.33	18.59	22.28	13.65	7.01	6.8	26.93	*
epha3 intron15	*	*	*	*	4.62	60.71	62.83	16.41	*	18.09	69.52	48.21	*	5.94	36.11	80.98	49.48	58.35
Antxr2	40.03	*	31.76	*	93.03	23.89	77.56	13.98	44.91	62.88	24.71	76.26	*	20.62	39.87	39.18	39.6	50.45
epha3 intron 5	*	*	*	74.63	*	*	18.1	12.72	*	27.01	*	34.51	68.37	20.6	50.5	*	40	63.68
COL24A1	*	*	*	*	34.37	96.44	65.91	5.68	*	83.55	95.76	23.28	*	15.56	21.69	*	16.32	*
ADMTS20	*	*	*	*	*	0	47.3	*	*	*	*	10.46	*	24.94	17.23	*	35.46	*
SPOCK3	*	15.76	4.11	13.88	14.04	4.08	6.71	6.76	8.45	14.4	0	38.79	6.3	23.41	*	12.37	11.1	8.56
MGC42174	*	11.2		32.28	18.29	*	*	*	*	42.91	*	*	*	*	28.01	*	*	21.39
LOC284395	23.68	54.77	62.69	10.76	7.77	*	17.26	13.75	*	43.67	55.2	52.83		6.32	15.37	*	35.32	8.87
FAM49A	43.28	24.67	*	7.6	*	10.04	45.15	34.72	26.79	92.86	*	51.33	72.84	35.29	40.03	*	22.02	*
LOC286094	38.13	*	*	*	42.48	58.54	75.01	6.25	*	65.69	68.83	64.42	*	17.15	48.38	*	19.21	63.29
LRP2	56.36	57.18	51.37	70.95	61.71	68.39	72.56	28.05	76.65	55.48	42.12	82.65	89.1	25.88	51.37	75.95	63.83	72.68
CDH8	*	*	*	96.81	46.23	84.54	75.89	52.23	90.46	60.35	*	50.56	*	19.27	62.35	*	92.05	58.46
CNTNAP5	62.19	47.22	39.65	49.81	49.74	43.11	57.8	38.11	45.75	27.48	70.34	34.76	38.3	15.23	41.12	62.94	40.16	31.09
LOC133993	58.4	*	*	*	*	82.56	*	33.01	87.88	*	76.8	99.29	*	34.54	*	66.2	*	*
PPP2R2B	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
PRKG1	88.81	*	*	84.56	79.75	*	85.59	87.67	*	94.27	79.06	77.52	*	74.67	74.43	*	77.09	85.05
COBRAL1	73.56	75.72	77.05	81.08	72	80.43	79.38	65.96	80.94	71.69	83.83	73.96	82.37	65.14	68.06	71.11	70.98	85.77

Table 4.6 : COBRA unique to L1 hypomethylation of Normal oral epithelial.

	Hypomethylation of Normal oral epithelial											
COBRA	Or1	Or2	Or3	Or4	Or5	Or6	Or7	Or8	Or9	Or10	Or11	Or12
PKP4	0	5.39	0	0	0	18.46	0	2.67	0	4.6	0.93	0
epha3 intron 15	3.41	6.26	1.04	14.77	3.85	3.12	8.97	7.45	1.8	1.45	1.61	6.03
Antxr2	3.53	2.37	5.68	9.42	3.37	6.3	2.89	2.33	7.83	6.05	7.76	4.1
epha3 intron 5	5.26	3.8	6.93	7.72	8.06	7.12	7.79	3.8	11.46	2.73	1.28	4.2
COL24A1	15.94	8.76	4.2	7.84	3.89	8.7	12.05	7.57	5.32	9.83	9.19	2.68
ADMTS20	11.8	7.64	14	9.34	10.73	6.88	9.8	10.65	10.8	7.22	10.58	8.34
SPOCK3	29.85	21.86	15.32	6.2	19.16	7.2	6.03	3.72	2.94	5.35	33.2	4.1
MGC42174	11.02	8.9	11.66	8.99	15.7	12.63	13.04	18.8	15.42	14.68	22.37	13.09
LOC284395	25.38	24.26	*	*	*	24.26	*	27.74	*	14.92	4.16	9.76
FAM49A	34.04	8.43	24.65	22.76	16.67	14.37	20.38	28.76	5.59	25.28	15.65	26.75
LOC286094	24	7.74	18.99	20.77	22.97	16.12	20.25	10.98	21.21	33.77	28.01	20.67
LRP2	23.79	22.26	20.16	19.04	11.35	30.29	5.65	10.64	48.23	24.91	21.4	26.07
CDH8	24.28	22.98	21.91	25.49	16.88	11.53	28.5	27.3	15.72	28.85	31.81	16.16
CNTNAP5	23.99	22.9	35.97	30.79	21.48	20.71	25.08	25.94	29.1	26.58	28.4	23.64
LOC133993	36.53	41.71	38.4	42.21	38.8	51.41	27.54	36.55	30.56	46.68	44.08	54.49
PPP2R2B	59.53	58.05	59.62	62.32	56.85	64.07	57.9	61.63	65.03	58.88	59.76	58.08
PRKG1	69.5	67.64	76.08	78.09	75.52	79.1	71.63	82	77.91	71.29	81.74	76.44
COBRAL1	58.62	51.93	59.29	56.32	57.96	51.67	59.44	58.91	52.48	60.19	63.17	62.04

Table 4.7 : COBRA unique to L1 hypomethylation of Normal white blood cell.

	Hypomethylation of Normal White Blood Cells											
COBRA	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
PKP4	2.99	4.46	2.5	3.96	0.5	0	2.33	3.69	3.33	4.5	0	3.77
epha3 intron 15	5.97	5.88	3.5	4.9	8.54	9.94	5.85	5.8	5.13	8.33	2.14	5.4
Anbxr2	5.07	4.24	1.13	4.93	3.67	2.56	2.06	1.38	3.35	4.6	5.84	5.84
epha3 intron 5	3.56	4.16	4.73	0	3.45	4.32	6.01	2.88	7.44	8.59	0	7.77
COL24A1	2.6	6.54	4.68	3.3	4.24	2.81	3.65	2.85	3.2	3.49	5.5	11.69
ADMTS20	12.14	5.67	3.39	12.4	4.11	8.89	3.73	5.2	4.56	9.2	1.17	3.56
SPOCK3	21.32	21.57	47.91	8.65	44.82	8.68	17.69	10.34	13.69	43.15	38.92	45.99
MGC42174	5.52	24.93	34.05	4.1	36.79	7.91	6.04	5.55	5.86	4.44	6.14	6.94
LOC284395	*	*	5.44	*	7.73	*	*	*	45.46	14.4	*	*
FAM49A	0.8	8.22	8.12	3.07	9.28	5.94	6.03	2.96	3.2	2.99	3.61	6.38
LOC286094	10.62	3.69	6	10.39	7.17	11.49	5.49	8.88	8.33	8.84	4.27	5.27
LRP2	3.79	2.71	2.15	1.64	4.15	3.31	2.82	3.61	3.11	3.76	3.57	3.82
CDH8	19.94	27.16	33.27	21.42	24.77	19.55	24.22	27.83	23.7	24.19	21.42	38.09
CNTNAP5	3.12	4.33	4.26	0	3.82	2.6	*	7.72	4.5	5.23	0	12.29
LOC133993	30.05	36.27	34.33	30.79	33.16	30.1	31.39	37	26.24	33.06	31.57	32.35
PPP2R2B	56.45	56.67	55.13	53.38	56.45	60.62	64.9	62.16	63.28	52.57	56.77	61.68
PRKG1	63.11	56.95	55.12	58.31	53.96	57.79	61.86	62.7	58.9	56.12	62.61	54.72
COBRAL1	53.78	52.09	51.71	53.78	54.38	53.63	51.26	55.38	55.79	55.01	54.29	55.12

Table 4.8 : Conclusion table for show COBRA unique to L1 each loci significant value from multiple group comparison with ANOVA analysis.

Compare groups/COBRA unique to LINE-1 loci		PKP4	EPHA3 intron 15	ANTXR2	EPHA3 intron 5	COL24A1	ADAMTS20	SPOCK3	MGC42174	LOC284395	FAM49A	LOC286094	LRP2	CDH8	CNTNAP5	LOC133993	PPP2R2B	PRKG1
HNSCC cell line	Leukemic cell line	0.979	0.270	0.001	0.786	0.253	0.025	0.989	0.298	0.163	0.039	0.000	0.000	0.064	0.002	0.004	0.459	0.001
HNSCC cell line	Epithelial cell line	0.998	0.637	0.731	0.112	0.359	0.322	0.335	0.187	0.015	0.122	0.963	0.196	0.299	0.942	0.265	0.470	0.025
HNSCC cell line	HNSCC microdissect cell	0.000	0.294	0.340	0.487	0.635	0.021	0.125	0.792	0.002	0.001	0.000	0.041	0.001	0.000	0.040	*	0.000
HNSCC cell line	Normal oral rinse cell	0.841	0.003	0.000	0.002	0.000	0.000	0.201	0.105	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
HNSCC cell line	Normal white blood cell	0.840	0.003	0.000	0.002	0.000	0.000	0.460	0.075	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Leukemic cell line	Epithelial cell line	0.986	0.660	0.006	0.246	0.927	0.013	0.420	0.737	0.191	0.719	0.001	0.034	0.588	0.014	0.195	0.245	0.528
Leukemic cell line	HNSCC microdissect cell	0.011	0.717	0.006	0.435	0.436	0.820	0.286	0.465	0.337	0.806	0.123	0.003	0.568	0.739	0.216	*	0.234
Leukemic cell line	Normal oral rinse cell	0.908	0.001	0.801	0.047	0.077	0.426	0.357	0.908	0.134	0.089	0.320	0.337	0.000	0.197	0.044	0.047	0.001
Leukemic cell line	Normal white blood cell	0.908	0.002	0.720	0.037	0.046	0.275	0.610	0.815	0.175	0.005	0.029	0.006	0.000	0.002	0.003	0.024	0.000
Epithelial cell line	HNSCC microdissect cell	0.025	0.844	0.351	0.044	0.553	0.013	0.057	0.305	0.431	0.517	0.007	0.918	0.252	0.007	0.728	*	0.073
Epithelial cell line	Normal oral rinse cell	0.901	0.016	0.001	0.667	0.091	0.000	0.079	0.769	0.813	0.053	0.000	0.001	0.000	0.000	0.001	0.003	0.000
Epithelial cell line	Normal white blood cell	0.901	0.019	0.000	0.594	0.058	0.000	0.618	0.851	0.940	0.004	0.000	0.000	0.000	0.000	0.000	0.001	0.000
HNSCC microdissect cell	Normal oral rinse cell	0.000	0.000	0.000	0.000	0.001	0.228	0.863	0.284	0.381	0.037	0.001	0.000	0.000	0.015	0.000	*	0.002
HNSCC microdissect cell	Normal white blood cell	0.000	0.000	0.000	0.000	0.000	0.123	0.022	0.228	0.458	0.000	0.000	0.000	0.000	0.000	0.000	*	0.000

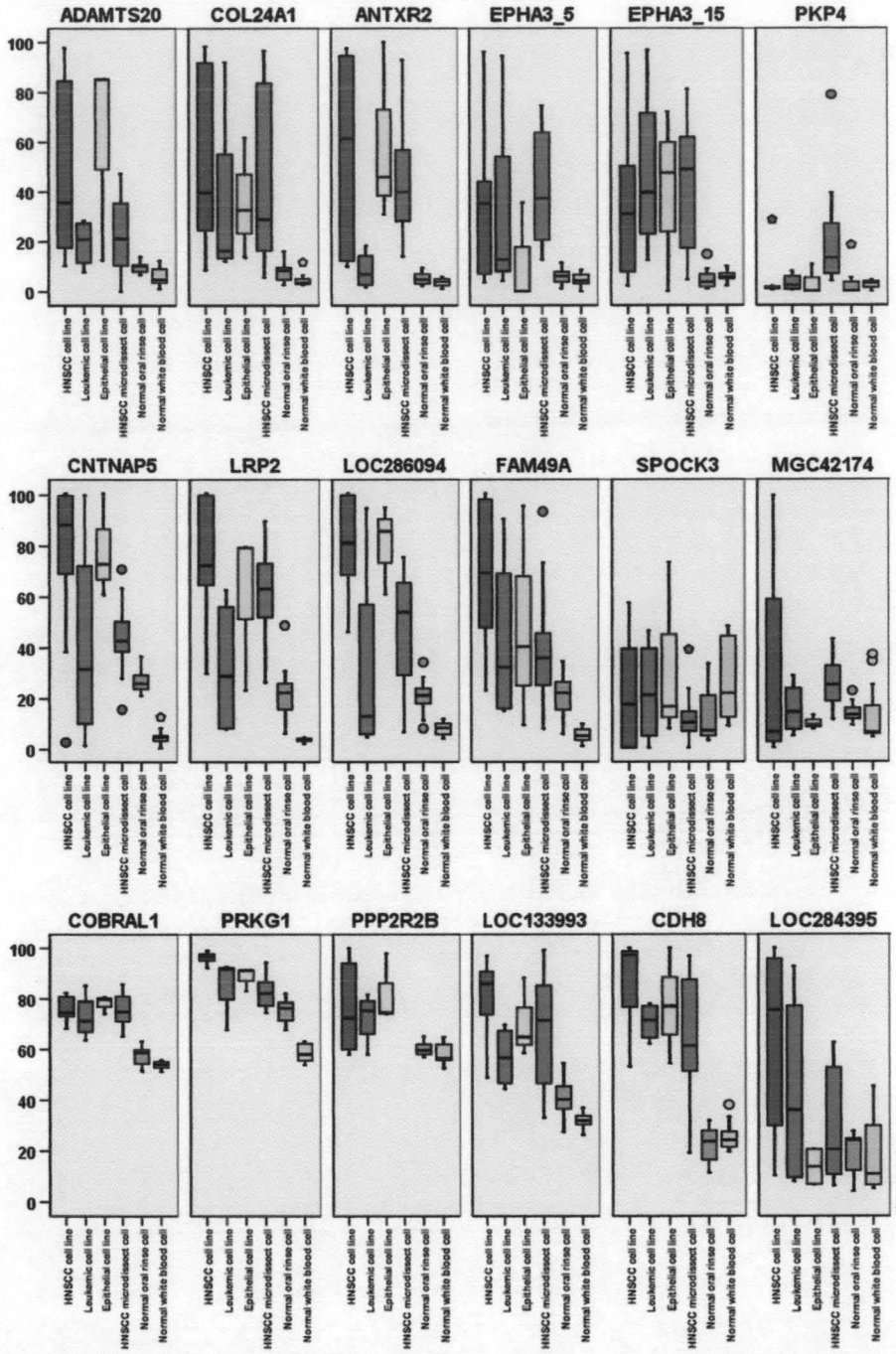

Figure 4.6 : Mean of hypomethylation compare among each cell type within all COBRA unique to L1 loci include COBRA L1.

Some of CU-L1 loci methylation level are normal tissue specific.

CU-L1 methylation levels of 9 loci were different between normal oral epithelial and normal white blood cell (Fig. 4.7 and Table 4.9). 8 CU-L1s, PRKG1, LOC133993, CNTNAP5, LRP2, LOC286094, FAM49A, ADAMTS20 and COL24A1, demonstrated higher hypomethylationlevel in normal oral epithelial than in normal white blood cell, which are. SPOCK3 is the single loci in indicating normal tissue differentiation with high loss of methylation in Normal white blood cell.

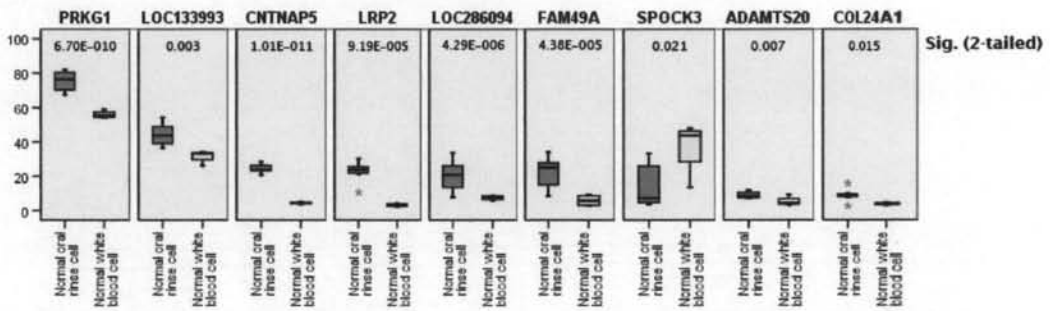

Figure 4.7 : Mean of hypomethylation compare between Normal white blood cell and Normal oral epithelial cell include all COBRA unique to L1 loci and COBRA L1.

Table 4.9 : Independent T-test for equality of means between Normal white blood cell and Normal oral epithelial cell.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
CNTNAP5	Equal variances assumed	.964	.337	13.337	21	.000	21.86318	1.63932	18.45402	25.27234
	Equal variances not assumed			13.475	20.607	.000	21.86318	1.62245	18.48519	25.24118
ANTXR2	Equal variances assumed	3.005	.097	1.692	22	.105	1.41333	.83515	-.31866	3.14533
	Equal variances not assumed			1.692	19.552	.106	1.41333	.83515	-.33132	3.15799
FAM49A	Equal variances assumed	11.046	.003	5.980	22	.000	15.22750	2.54648	9.94643	20.50857
	Equal variances not assumed			5.980	13.153	.000	15.22750	2.54648	9.73267	20.72233
COL24A1	Equal variances assumed	1.468	.239	2.654	22	.015	3.45167	1.30064	.75430	6.14903
	Equal variances not assumed			2.654	19.424	.015	3.45167	1.30064	.73342	6.16992
ADAMTS20	Equal variances assumed	4.757	.040	3.027	22	.006	3.64667	1.20472	1.14823	6.14510
	Equal variances not assumed			3.027	17.472	.007	3.64667	1.20472	1.11015	6.18319
LOC284395	Equal variances assumed	2.160	.176	.047	9	.964	.38250	8.15552	-18.06658	18.83158
	Equal variances not assumed			.039	3.842	.971	.38250	9.87864	-27.49539	28.26039
LOC286094	Equal variances assumed	2.931	.101	6.054	22	.000	12.92000	2.13397	8.49443	17.34557
	Equal variances not assumed			6.054	14.060	.000	12.92000	2.13397	8.34494	17.49506
LRP2	Equal variances assumed	8.734	.007	5.953	22	.000	18.77917	3.15452	12.23709	25.32125
	Equal variances not assumed			5.953	11.104	.000	18.77917	3.15452	11.84405	25.71428
CDH8	Equal variances assumed	.593	.449	-1.178	22	.251	-2.84583	2.41591	-7.85613	2.16446
	Equal variances not assumed			-1.178	21.615	.252	-2.84583	2.41591	-7.86131	2.16964
LOC133993	Equal variances assumed	7.044	.014	3.546	22	.002	8.55417	2.41250	3.55094	13.55740
	Equal variances not assumed			3.546	13.988	.003	8.55417	2.41250	3.37945	13.72888
PKP4	Equal variances assumed	3.094	.092	.001	22	.999	.00167	1.61314	-3.34379	3.34712
	Equal variances not assumed			.001	13.095	.999	.00167	1.61314	-3.48075	3.48408
MGC42174	Equal variances assumed	11.557	.003	.408	22	.687	1.50250	3.67916	-6.12761	9.13261
	Equal variances not assumed			.408	13.249	.690	1.50250	3.67916	-6.43068	9.43568
PPP2R2B	Equal variances assumed	4.876	.038	1.306	22	.205	1.80500	1.38208	-1.06125	4.67125
	Equal variances not assumed			1.306	18.705	.207	1.80500	1.38208	-1.09081	4.70081
PRKG1	Equal variances assumed	1.160	.293	10.327	22	.000	17.06583	1.65249	13.63878	20.49288
	Equal variances not assumed			10.327	19.931	.000	17.06583	1.65249	13.61804	20.51363
SPOCK3	Equal variances assumed	6.322	.020	-2.521	22	.019	-13.98333	5.54654	-25.48616	-2.48051
	Equal variances not assumed			-2.521	19.276	.021	-13.98333	5.54654	-25.58113	-2.38554
EPHA3_15	Equal variances assumed	3.915	.061	-.736	22	.469	-.96833	1.31480	-3.69506	1.75839
	Equal variances not assumed			-.736	16.839	.472	-.96833	1.31480	-3.74434	1.80767
EPHA3_5	Equal variances assumed	.153	.699	1.258	22	.221	1.43667	1.14166	-.93100	3.80433
	Equal variances not assumed			1.258	21.982	.221	1.43667	1.14166	-.93111	3.80444

L1 methylation lost in cancer is not generalized.

L1 loss of methylation during cancer progression is a common epigenetic event. Here, we evaluated if the epigenetic event occurred equally throughout the cancer genome. Noticeably in figure 4.5, HN6, 19, 26 and KB possessed high degree of CU-L1 hypomethylation at ANTXR2 but hypermethylated at SPOCK3. The random pattern of L1 hypomethylation can be discovered in other HN cells as well as microdissected HNSCC. Although the number of loci with hypomethylation were in direct correlation with COBRA-L1 (Fig. 4.8). Note worthily, EPHA3 CU-L1 loci is the eminent loss of methylation during cancer progression. ANTXR2 CU-L1loci and FAM49A CU-L1 loci are favorite location in gain methylation during metastasis. Normal oral epithelial group has narrow low level of hypomethylation, however HNSCC cell line group have variety pattern of hypomethylation among CU-L1 specific loci. The sporadic and distinctive pattern of L1 hypomethylation loci may be resulted from selective expansion of a clone that lost L1 at the particular loci. HN19 is the obvious sample, selective low hypomethylation at COL24A1 loci and highly loss of methylation at EPHA3 intron 5 loci. Therefore, these CU-L1 experiments indicated possible carcinogenic potential differences of loss of DNA methylation among L1 loci.

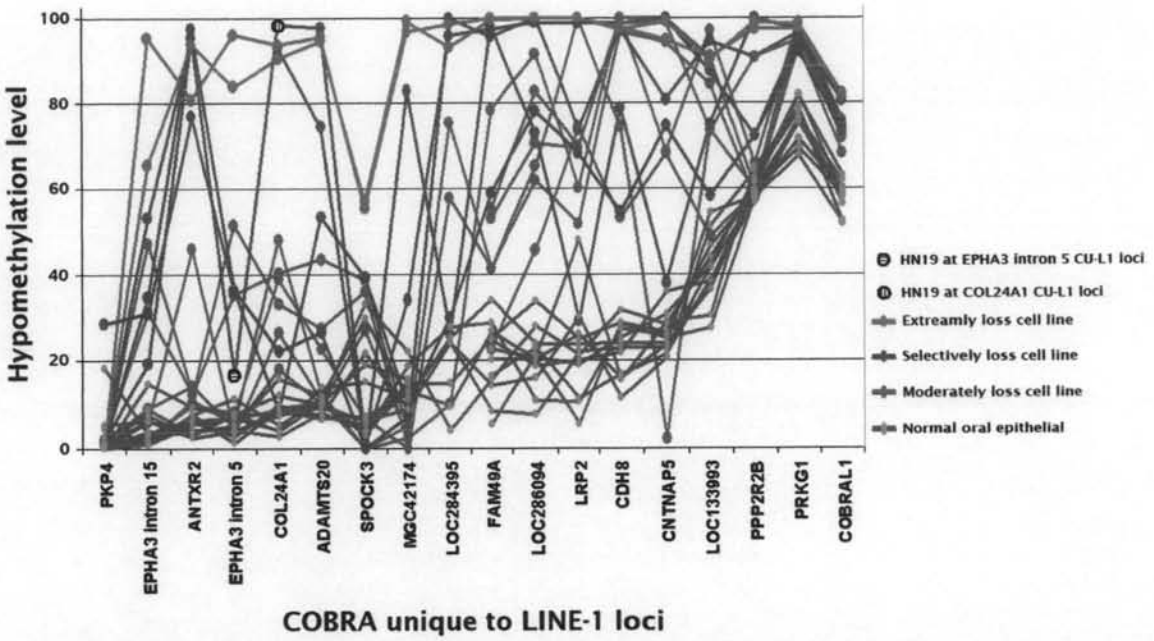

Figure 4.8 : Demonstration character of HNSCC including Selection L1 loci in loss of methylation process during cancer progression and Clonal expansion. Extreamly loss cell line group include HN30 and HN31, which have high hypomethylation level of all CU-L1. Selectively loss cell line mean to HN6, HN19, HN26 and KB, which have specific selection CU-L1 loci for high loss of methylation. Moderately loss cell include HN4, HN8, HN12, HN13, HN17 and HN22, hypomethylation level of them are not extream but higher than normal oral epithelial group.

Influence of L1 promoter hypomethylation to host gene expression

The differences of CU-L1 level among loci between normal tissues as well as the characteristic of selective clonal expansion in HN cells indicated that L1 methylation may possess biological role in cis. We hypothesized that L1 methylation may be associated with the level of their linked genes. Because all of our evaluated L1s are within intron, we measured the expression level of all 17 genes and. In figure 4.9 the gel electrophoresis of RT-PCR of EPHA3 and GAPDH was shown, Θ is negative control of PCR, R is RNA for cDNA quantitated control and C is amplification of RT-PCR from cDNA of each cell line, which represent name in number. Gene expression of host per GAPDH ratio are present in table 4.10. While most did not show correlation, *EPHA3* and *PPP2R2B* did. Data demonstrating *EPHA3* intron 5 and 15 CU-L1 loci loss of methylation level associate with *EPHA3* expression was showed in figure 4.10 and 4.11 respectively, and PPP2R2B correlation was display in figure 4.12. This result indicated that L1 methylation may play a role in gene regulation. Interestingly, whereas promoter methylation is usually inhibit gene expression, down regulation of EPHA3 and PPP2R2B were observed when the intronic L1s were hypomethylated. It is interesting to further explore the underlining mechanisms of this epigenetic control and if the phenomenon is discoverable in other loci.

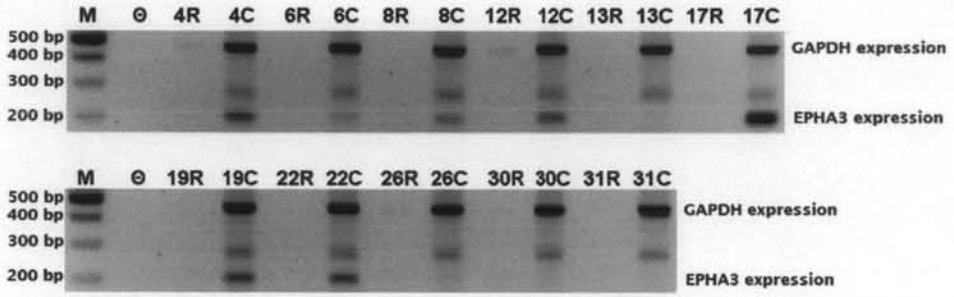

Figure 4.9 : Gel electrophoresis of RT-PCR for detect EPHA3 expression compare with GAPDH expression.

Table 4.10 : Ratio expression of L1 host gene per GAPDH expression

type	HN4	HN6	HN8	HN12	HN13	HN17	HN19	HN22	HN26	HN30	HN31
ANTXR2	0.020611	0.016226	0.013359	0.017811	0.016594	0.020252	0.016736	0.015524	0.017493	0.016081	0.013987
FAM49A	0.017074	0.005193	0.010464	0.00258	0.004876	0.001679	0.004765	0.008878	0.010542	0.002807	0.010294
COL24A1	0.003122	0.003804	0	0	0	0.003407	0.008111	0	0	0	0
ADAMTS20	0	0	0	0	0	0	0.012829	0.00536	0	0.003178	0
LRP2	0	0	0.005302	0	0	0.009471	0.013716	0	0	0	0
CDH6	0.015078	0	0.018139	0.00688	0	0.008019	0.017457	0.007994	0.014301	0.012311	0.005578
PKP4	0.052091	0.042441	0.038075	0.0408	0.032629	0.027227	0.029878	0.034537	0.039703	0.033511	0.043467
MGC42174	0.017704	0.017095	0.015567	0.019074	0.019973	0.016726	0.021056	0.021615	0.017428	0.024214	0.022551
PPP2R2B	0.034526	0	0.031171	0.015464	0.033739	0.019014	0.021935	0.029351	0.017077	0	0
PRKG1	0	0	0.006775	0	0	0	0.00878	0	0	0	0
SPOCK3	0.040907	0	0.001999	0.00951	0	0	0.008129	0.012285	0.008262	0.004836	0
EPHA3	0.756428	0.18448	0.398898	0.609248	0	1.39617	0.663149	0.673881	0.027733	0	0

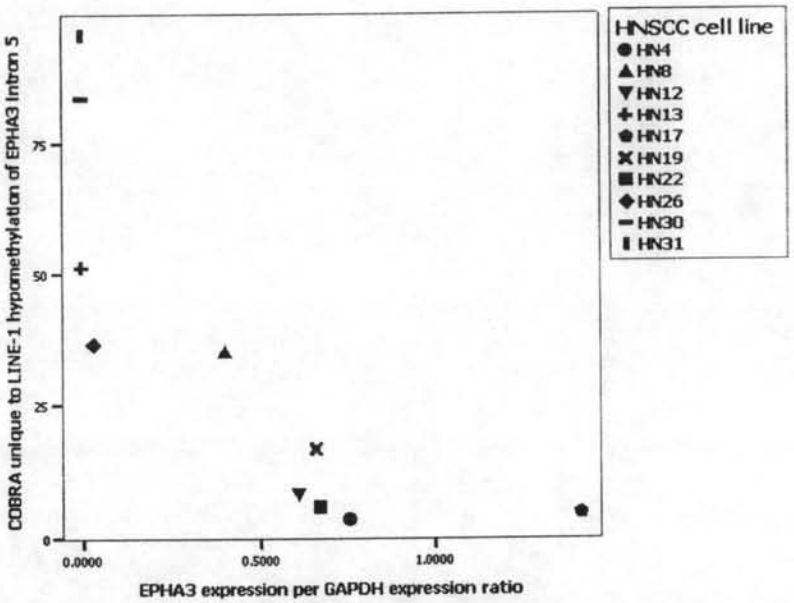

Figure 4.10 : Graph show linear correlation between EPHA3 expression and COBRA unique to L1 in EPHA3 intron 5 hypomethylation.

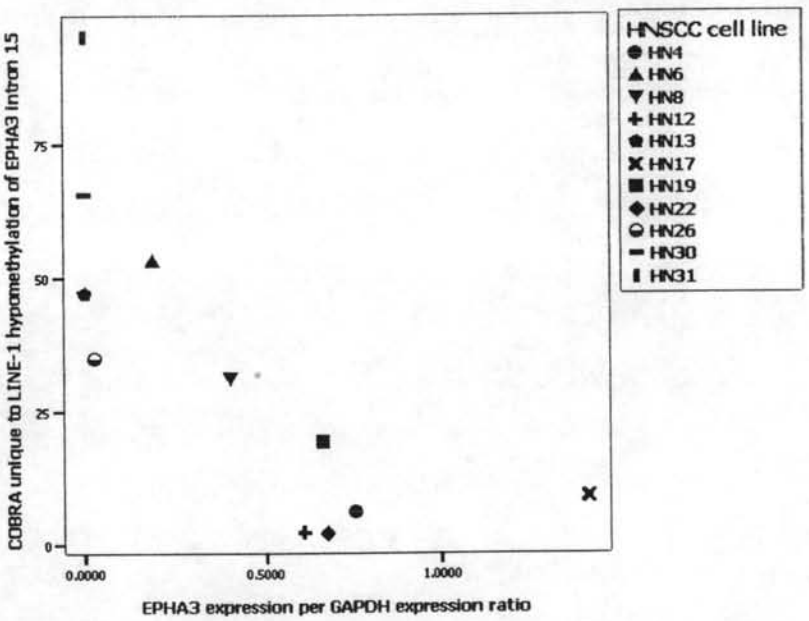

Figure 4.11 : Graph show linear correlation between EPHA3 expression and COBRA unique to L1 in EPHA3 intron 15 hypomethylation.

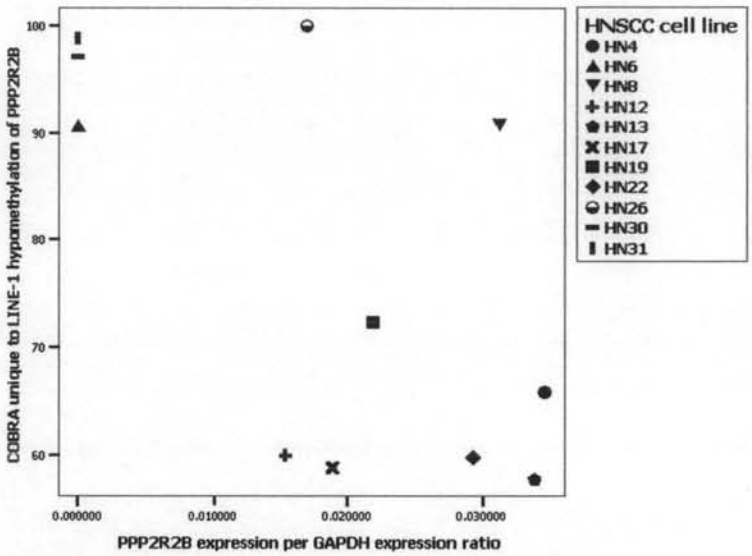

Figure 4.12 : Graph show linear correlation between PPP2R2B expression and COBRA unique to L1 in PPP2R2B intron 8 hypomethylation.

Table 4.11 : Pearson correlation of 13 L1 host gene expression and COBRA unique to L1 hypomethylation.

Cell type	Pearson correlaition	Sig(2-tailed)
ANTXR2	-0.1168	0.7323
FAM49A	0.228	0.5002
COL24A1	0.3343	0.315
ADAMTS20	0.4218	0.1963
LRP2	0.4016	0.2209
CDH8	-0.1941	0.5673
PKP4	-0.4564	0.1582
MGC42174	0.5799	0.0615
PPP2R2B	-0.6295	0.038
PRKG1	-0.438	0.1779
SPOCK3	-0.4737	0.141
EPHA3 intron 15	-0.7237	0.0118
EPHA3 intron 5	-0.7033	0.0233