Supplemental Material to:

Priscilla Brebi, Leonel Maldonado, Maartje G Noordhuis, Carmen Ili, Pamela Leal, Patricia Garcia, Mariana Brait, Judith Ribas, Christina Michailidi, Jimena Perez, Ethan Soudry, Oscar Tapia, Pablo Guzman, Sergio Muñoz, Leander Van Neste, Wim Van Criekinge, Rafael Irizarry, David Sidransky, Juan C Roa, and Rafael Guerrero-Preston

Genome-wide methylation profiling reveals Zinc finger protein 516 (ZNF516) and FK-506-binding protein 6 (FKBP6) promoters frequently methylated in cervical neoplasia, associated with HPV status and ethnicity in a Chilean population

Supplementary Table 1. Characteristics of patients that participated in the study

	Overall n=294		Normal n=37		CIN n=137		Cancer n=120	
Age								
Median	41		39		36		51	
Range	18-86		18-79		18-68		23-86	
	n	(%)	n	(%)	n	(%)	n	(%)
Ethnicity								
Non-Mapuche	223	(76%)	22	(59%)	112	(82%)	89	(74%)
Mapuche	71	(24%)	15	(41%)	25	(18%)	31	(26%)
HPV								
Not detected	60	(20%)	34	(92%)	17	(12%)	9	(8%)
Positive	234	(80%)	3	(8%)	120	(88%)	111	(93%)
HPV subtype								
HPV 16	155	(53%)	1	(3%)	70	(51%)	84	(70%)
HPV 18	52	(18%)	0	(0%)	25	(18%)	27	(23%)
Other HPV type	71	(24%)	2	(5%)	45	(33%)	24	(20%)

CIN=cervical epithelial neoplasia

Supplementary Table 2. Age, ethnicity and HPV status of patients selected for MeDIP-chip

	Normal n=12		Cancer n=7	
Age				
Median	37		49	
Range	24-53		31-63	
	n	*(%)*	*n*	*(%)*
Ethnicity				
Non-Mapuche	6	(50%)	3	(43%)
Mapuche	6	(50%)	4	(57%)
HPV				
Not detected	12	(100%)	0	(0%)
Positive	0	(0%)	7	(100%)
HPV subtype				
HPV 16	0	(0%)	6	(86%)
HPV 18	0	(0%)	3	(43%)
Other HPV type	0	(0%)	2	(29%)

Supplementary Table 3. Comparison of the Validation and Prevalence cohort

	Normal			Cancer		
	Validation cohort n=19	Prevalence cohort n=18	*P*-value	Validation cohort n=30	Prevalence cohort n=90	*P*-value
Age						
Median	36	41	0.28[1]	50	52	0.36[1]
Range	18-79	29-62		32-84	23-86	
	n (%)	*n* (%)		*n* (%)	*n* (%)	
Ethnicity						
Non-Mapuche	12 (63%)	10 (56%)	0.64[2]	27 (90%)	62 (69%)	**0.02[2]**
Mapuche	7 (37%)	8 (44%)		3 (10%)	28 (31%)	
Socio-economic status						
Indigent	7 (37%)	11 (61%)	0.33[2]	10 (33%)	52 (58%)	**0.02[2]**
≤USD310/month	8 (42%)	5 (28%)		16 (53%)	24 (27%)	
>USD310/month	4 (21%)	2 (11%)		4 (13%)	14 (16%)	
HPV						
Not detected	17 (89%)	17 (94%)	1.00[3]	2 (7%)	7 (8%)	1.00[2]
Positive	2 (11%)	1 (6%)		28 (93%)	83 (92%)	
HPV subtype						
HPV 16	0 (0%)	1 (6%)		22 (73%)	62 (69%)	
HPV 18	0 (0%)	0 (0%)		8 (27%)	19 (21%)	
Other HPV type	2 (11%)	0 (0%)		2 (7%)	22 (24%)	

1. Mann Whitney-U test; 2. Chi^2-test; 3. Fisher's exact test

Supplementary Table 4. Median Promoter methylation in premalignant cervical lesions.

GENE	Normal (n=37)		LGL (n=53)		HGL (n=84)		SCC n=120)	
	median	p-value	median	p-value	median	p-value	median	p-value
FKBP6	32.69	-	121.50	>0.01	79.65	>0.01	74.54	>0.01
FKBP6			121.50	-	79.65	>0.01	74.54	>0.01
FKBP6					79.65	-	74.54	0.1
INTS1	40.35		55.78	>0.01	48.07	>0.01	55.01	>0.01
INTS1			55.78	-	48.07	0.84	55.01	0.18
ZNF516	84.94	-	235.94	>0.01	136.42	>0.01	273.75	>0.01
ZNF516			235.94	-	136.42	>0.01	273.75	>0.01
ZNF516					136.42	-	273.75	>0.01

Supplementary Table 5. Distribution of gene promoter methylation across tissue types

Gene	Normal n=37		LSIL n=53		HSIL n=84		Cancer n=120	
	n/total	*(%)*	*n/total*	*(%)*	*n/total*	*(%)*	*n/total*	*(%)*
FKBP6 methylation	7/37	(19%)	51/53	(96%)	57/84	(68%)	68/109	(62%)
INTS1 methylation	9/36	(25%)	22/53	(42%)	22/82	(27%)	46/105	(44%)
ZNF516 methylation	1/36	(3%)	33/53	(62%)	28/76	(37%)	80/119	(67%)
SAP130 methylation	4/19	(21%)	ND	(ND)	ND	(ND)	0/30	(0%)
GGTLA4 methylation	10/19	(53%)	ND	(ND)	ND	(ND)	14/30	(47%)

LSIL - Low Grade Squamous Intraepithelial Lesion
HSIL - High Grade Squamous Intraepithelial Lesions
ND -Not Determined

Supplementary Table 6. Primer and probe list of genes used for bisulfite sequencing and (quantitative) Methylation Specific PCR

Gene name	Forward 5' – 3'	Reverse 5' – 3'	Probe 5'/56-FAM/-/ZEN/-/3IABkFQ/3'	Product (BP)	Tm (ºC)
BS-GGTLA4	GAGGTTTGTTTGTAGAGGTTC	CAAAACAACTCTAAAAAAATTTTC		397	52
BS-FKBP6	GTTTTAAAAGTGTTTTTTTTGTGTTT	GAACTCTAAAACTACAAAAACCAC		439	56
BS-ZNF516	GAGTGTTGTTGGTAGATTGTTG	CTATAAACAATACCAAACCTCAC		347	56
BS-SAP130	GGGAGGGGTGGGTTGATTC	GCTAACCCCACTCACCCCC		443	56
BS-INTS1	TTTTTTTTTGTAGTTTTATTTATAGC	CCAAAATCACTAAAAAAAAACAAAC		432	54
MSP-ZNF516 M	TACGACGGTGAGGTACGTATAC	CAAAAACACAAAAATAATACTCGAA	AACGCCAAACCTCACCGTCGTACG	241	54.2
MSP-ZNF516 U	GTATGATGGTGAGGTATGTATATGA	CAAAAACACAAAAATAATACTCAAA		242	50
MSP-FKBP6 M	TTACGTGTTTTATTATGTTTCGTGC	GAAAAAACACTCATCGTTTCGTT	CGACCCTAACCCTCGCGAACTCTA	137	58
MSP-FKBP6 U	ATGTGTTTTATTATGTTTTGTGTGT	AAAAAAACACTCATCATTTCATT		135	54
MSP-GGTLA4 M	TTGGATATTAAAGGGTGATTTTC	CCGTAATCCTACAAACCCTACG	ACGTCCTCCAACTCAACCACTCCA	183	55
MSP-GGTLA4 U	TTGGATATTAAAGGGGTGATTTTT	TTCCATAATCCTACAAACCCTACAT		185	52.5
MSP-SAP130 M	CGTTAGTTAATAGACGGGAGGTTC	CTAAATACTACGCCCAATAACCG	TCCCGCGCGCTCTCCGTCTATAAA	189	52.5
MSP-SAP130 U	TGTGTTAGTTAATAGATGGGAGGTTT	CCTAAATACTACACCCAATAACCAC		192	55
MSP-INTS1 M	CGAAGGGGTTGTTAGTAGTAGC	AAACAAAAAAAATAACCGACGAT	TATAACCTCCGCCCTCCCTCCCTA	143	55
MSP-INTS1 U	GTGAAGGGGTTGTTAGTAGTAGTGT	AAAAAACAAAAAAAATAACCAACAAT		147	52
β-actin	GTGTTTAGGGTTTTTTGTTTTTTTT	AACCACTCACCTAAATCATCTTCTC	ACCACCACCCAACACACAATAACAAACACA	280	58

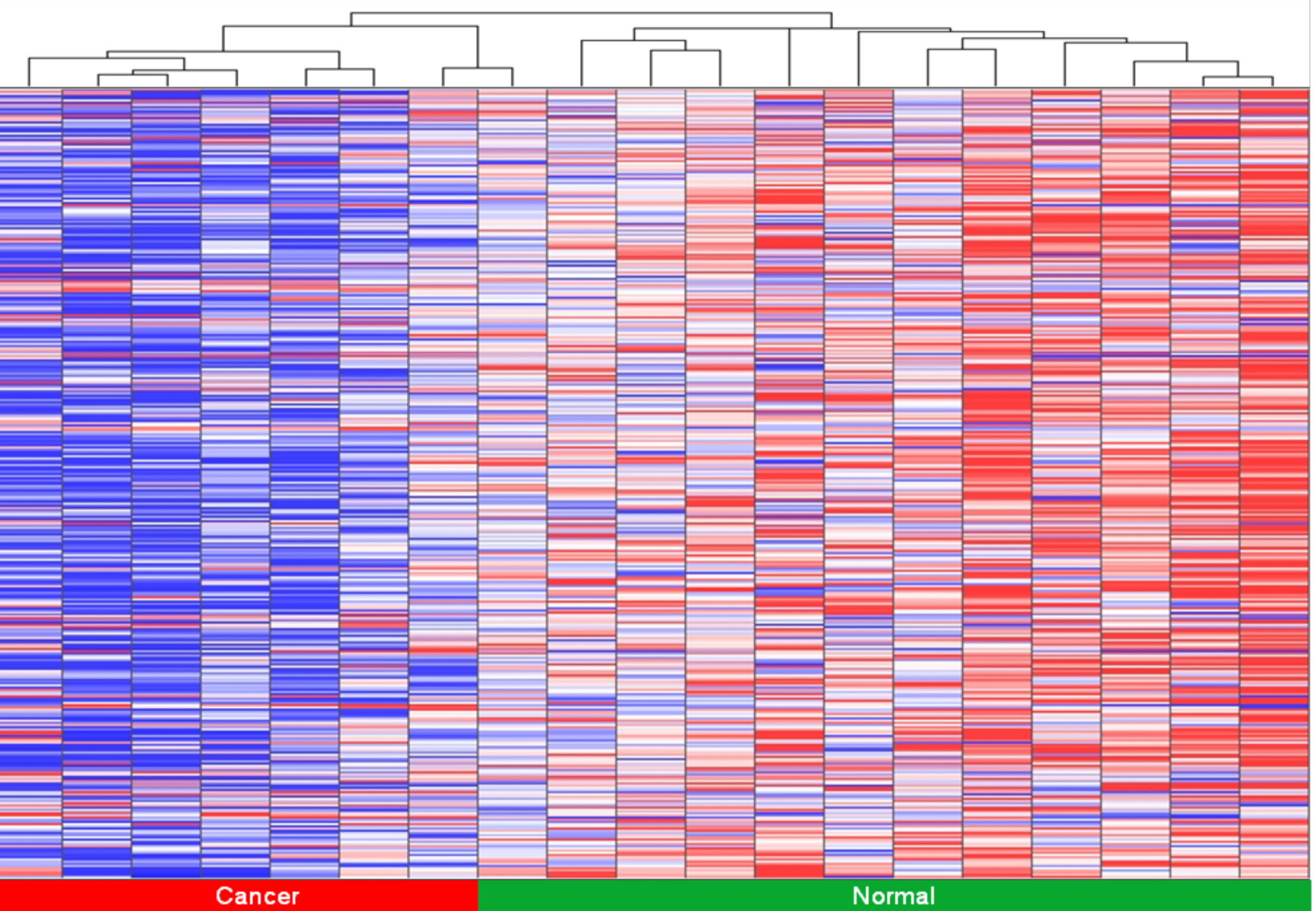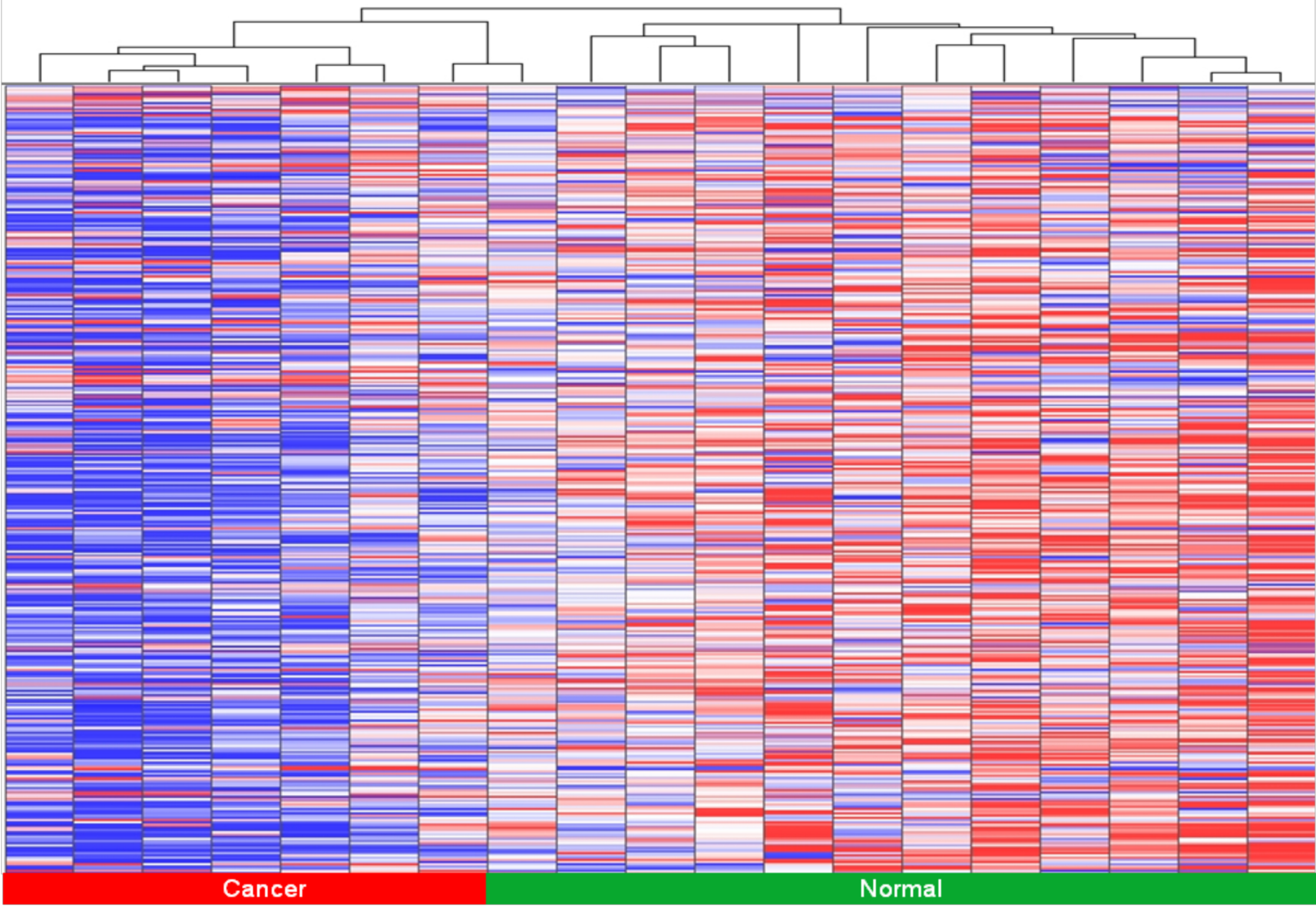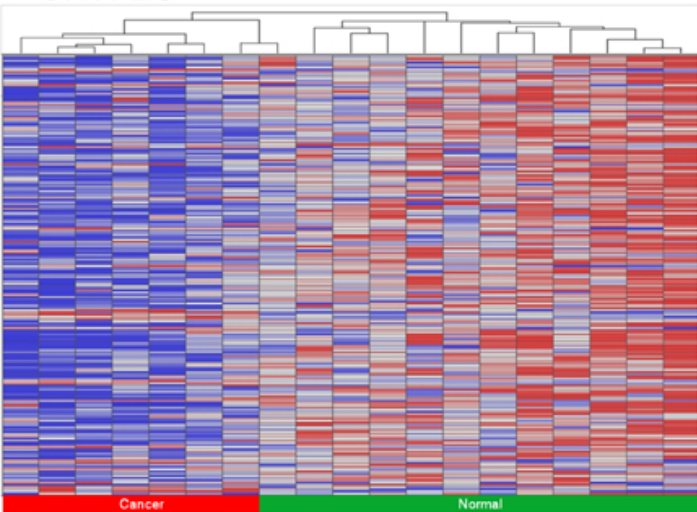BS: Bisulfite sequencing, MSP: Methylation Specific PCR, M: Methylated, U: Unmethylated, BP: base pairs, Tm: melting temperature.
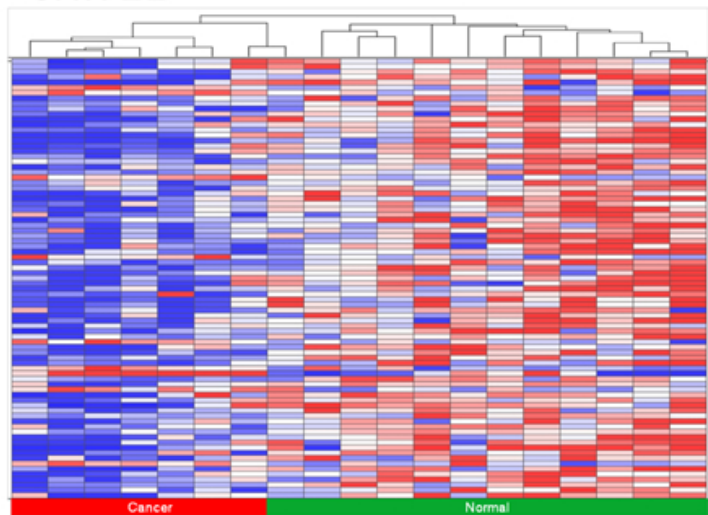
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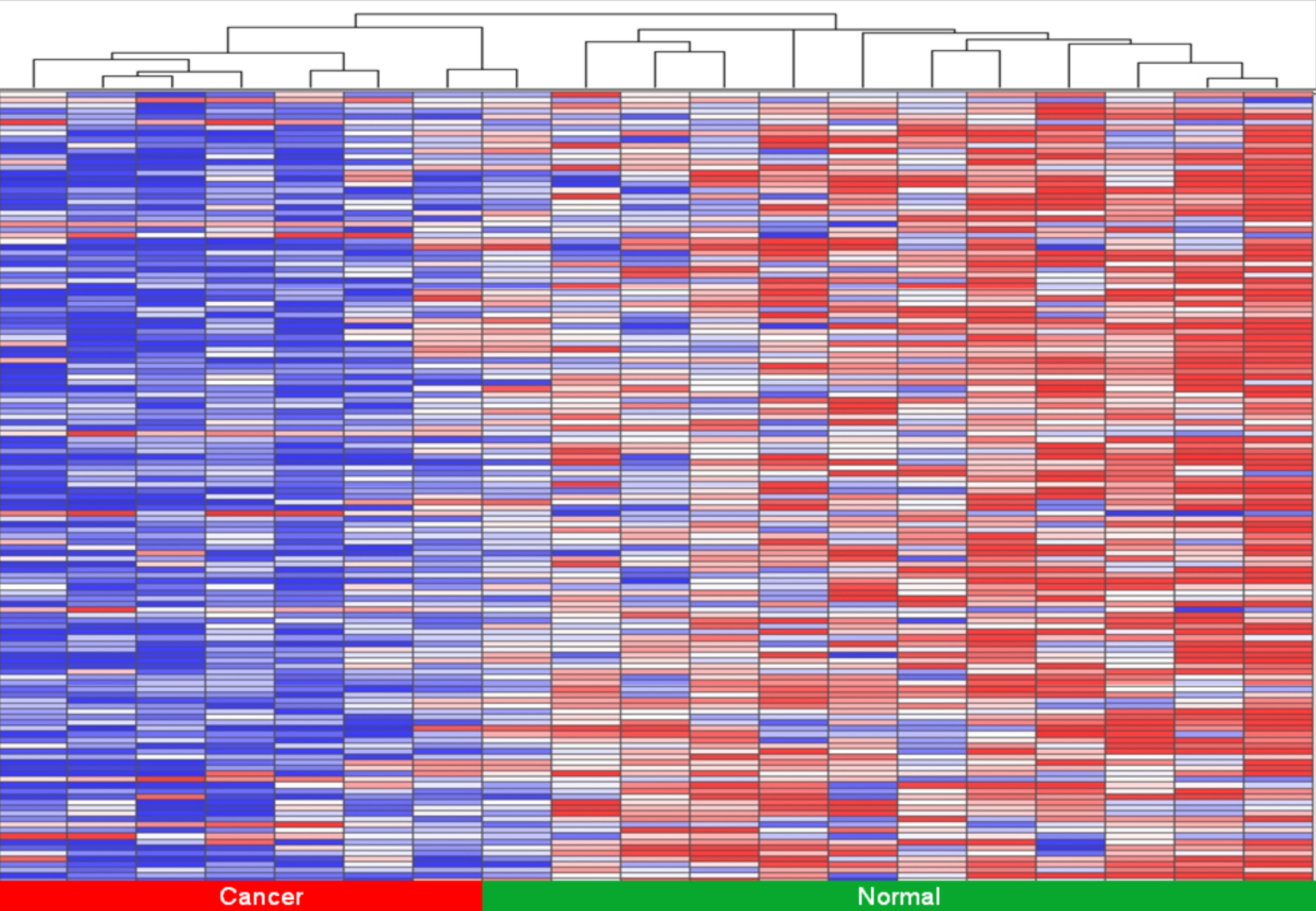
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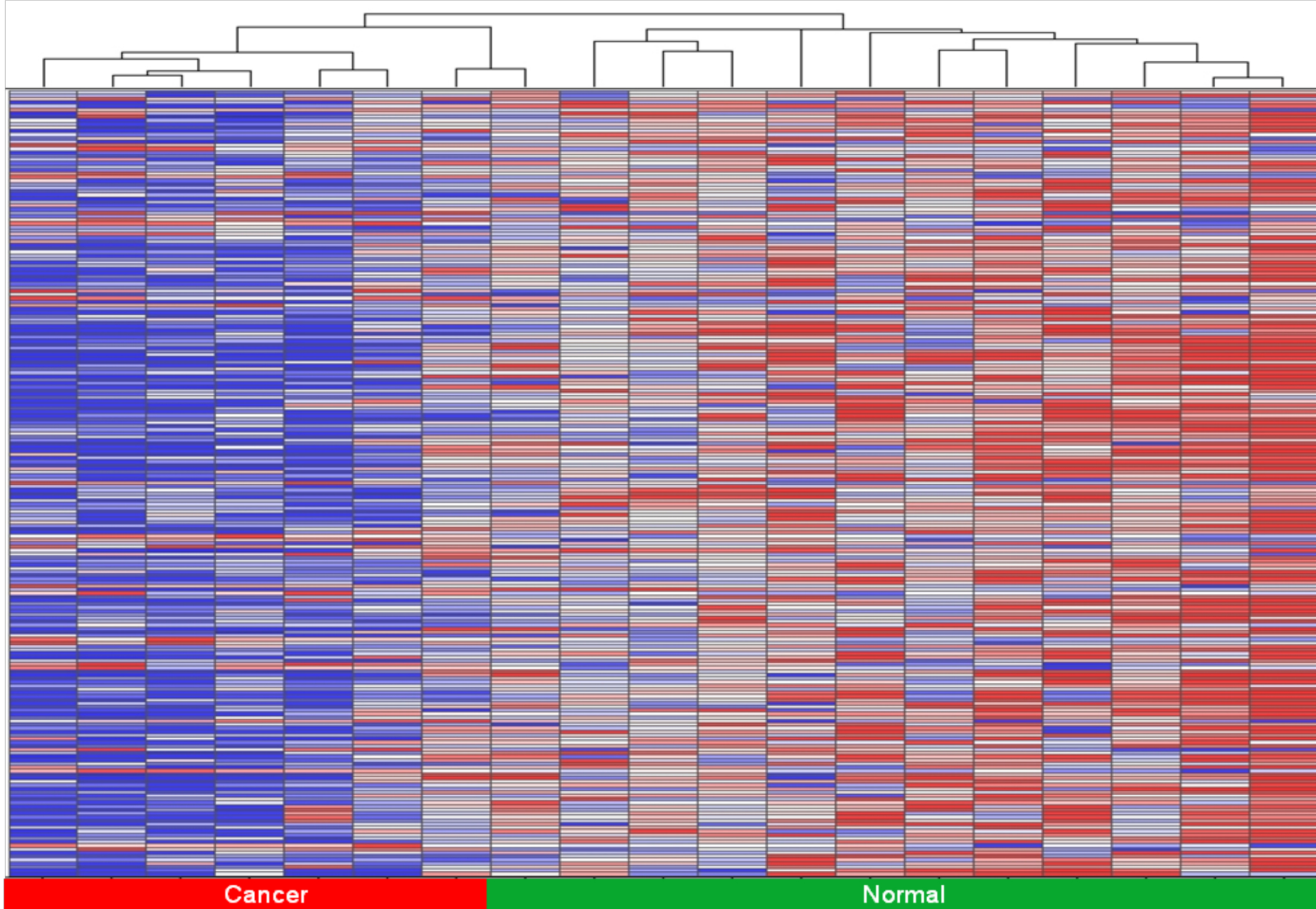
CHR 17

CHR 19

CHR 20

CHR 21

CHR 22
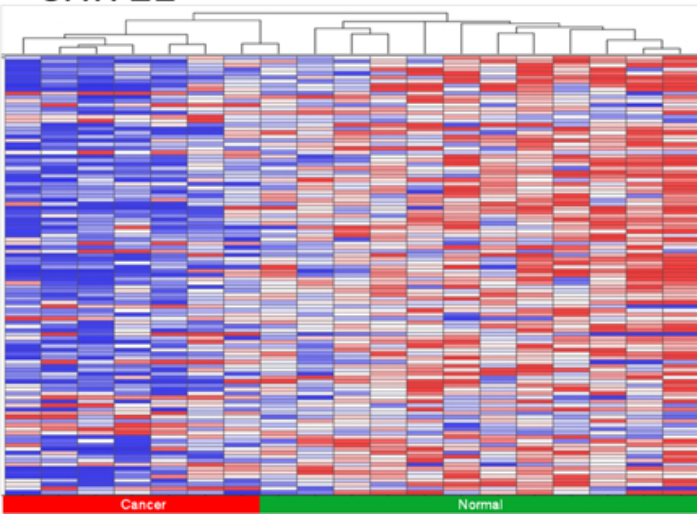
CHR X
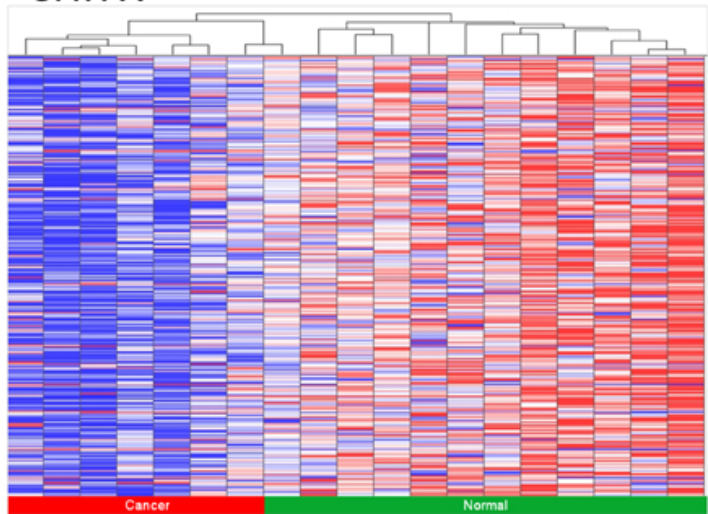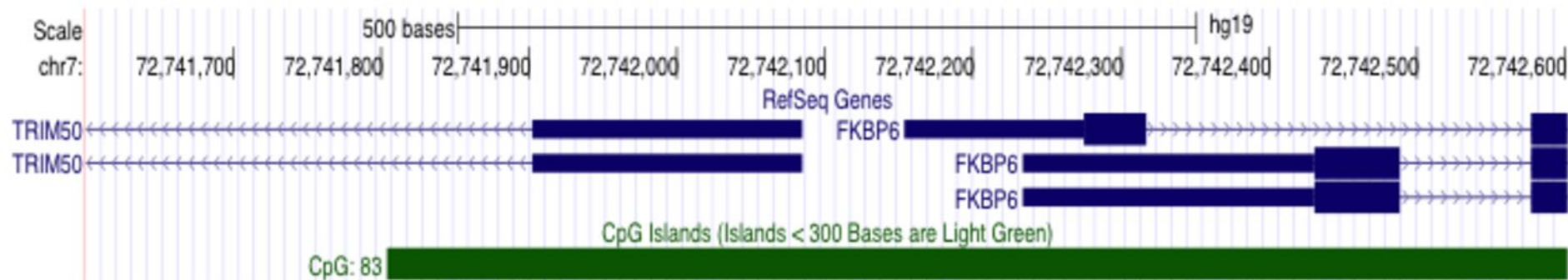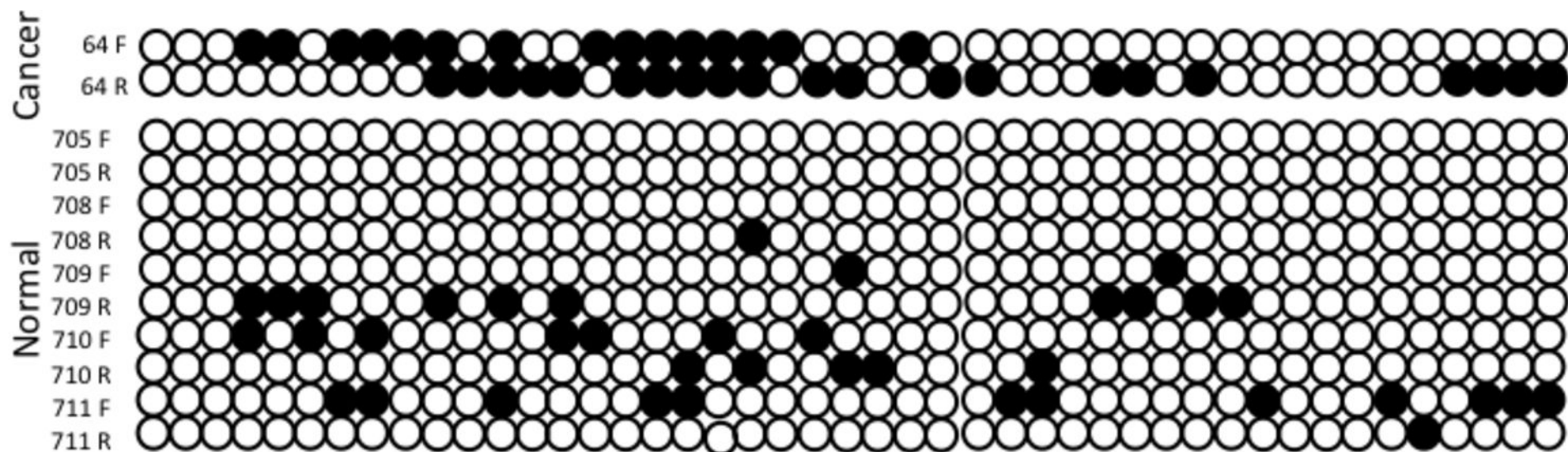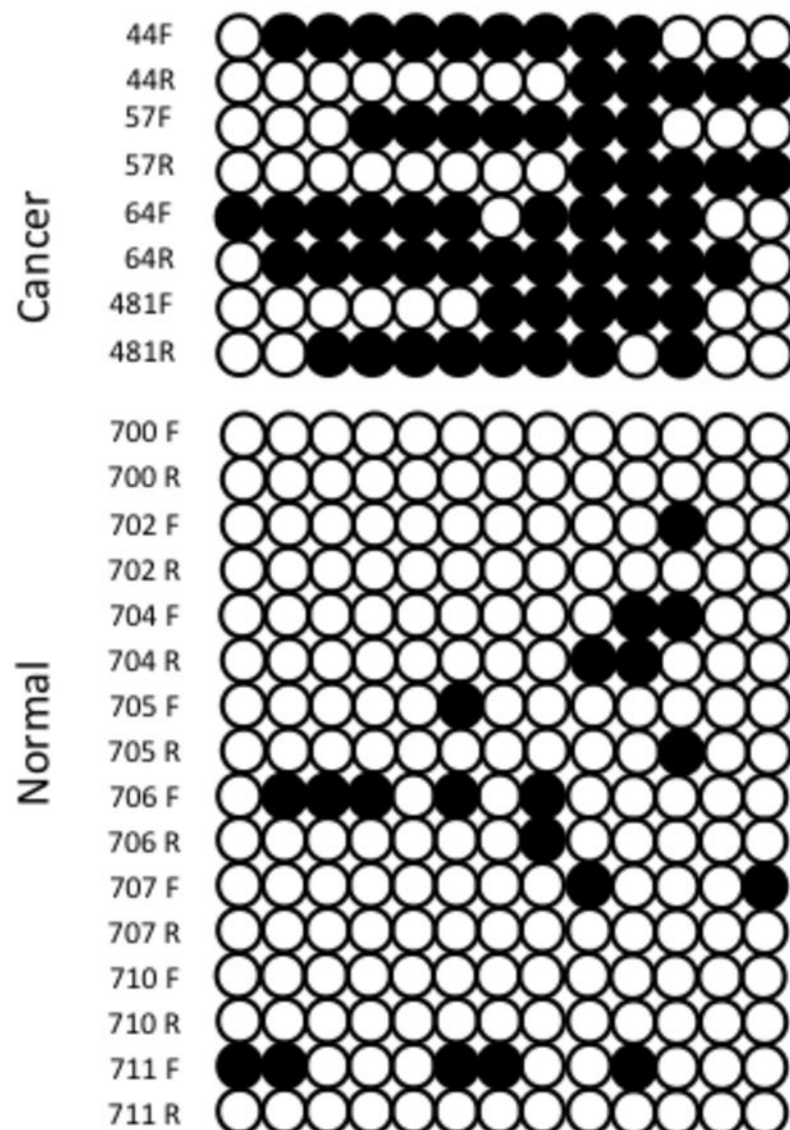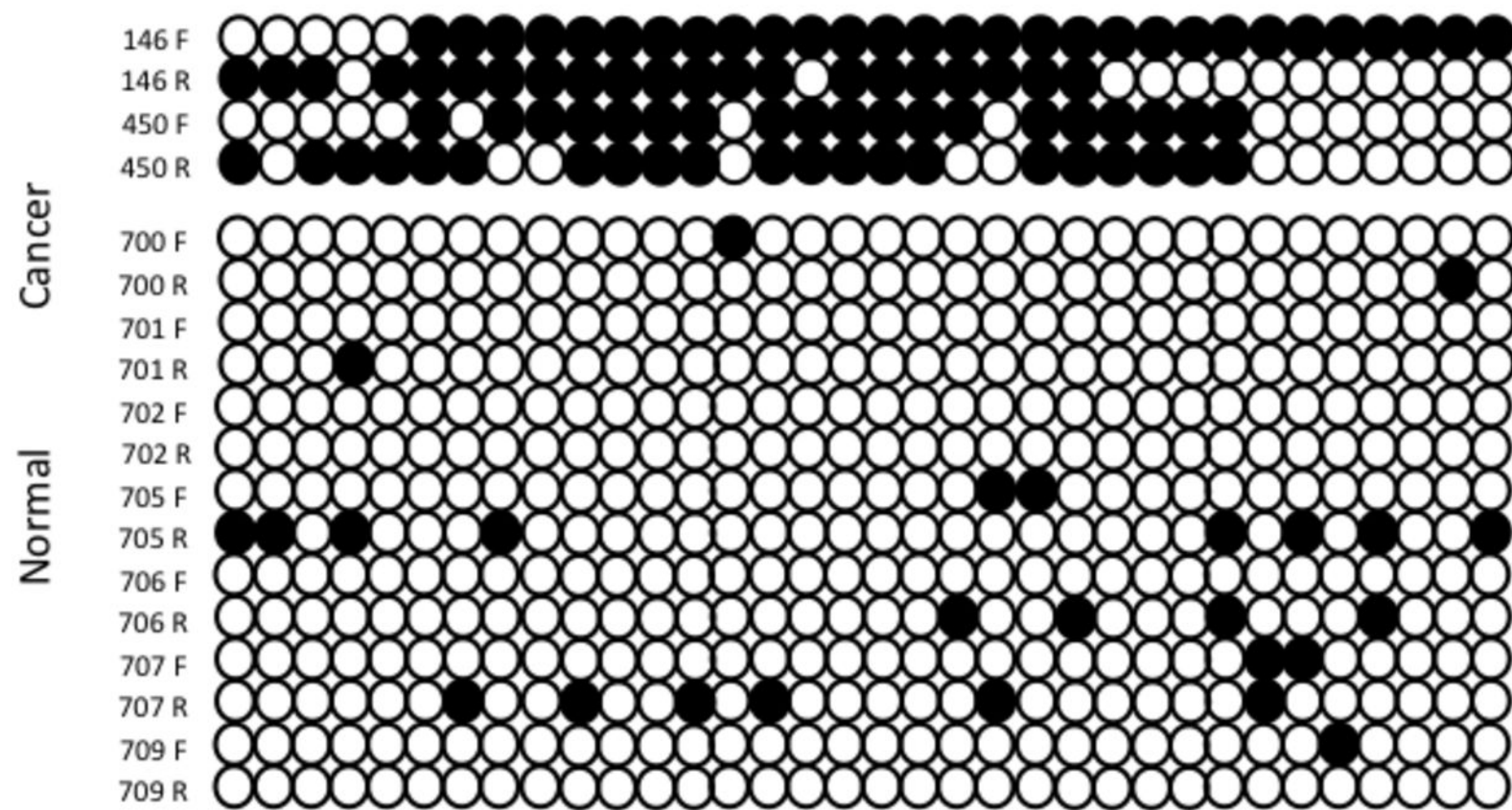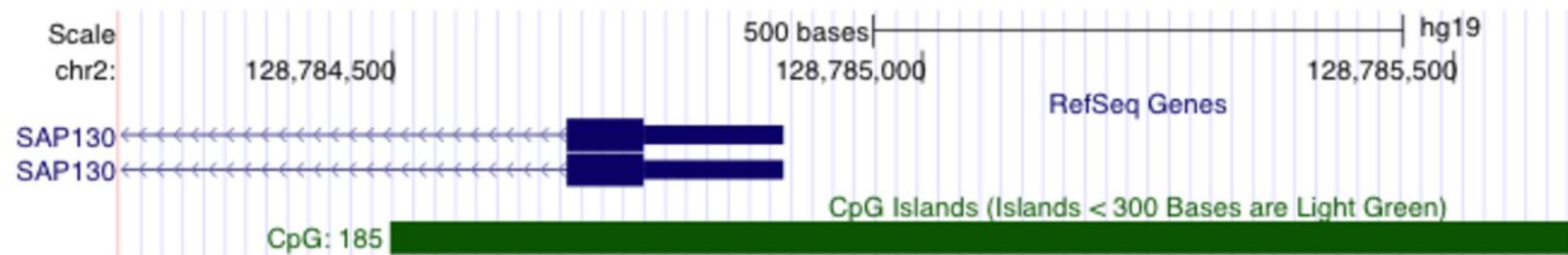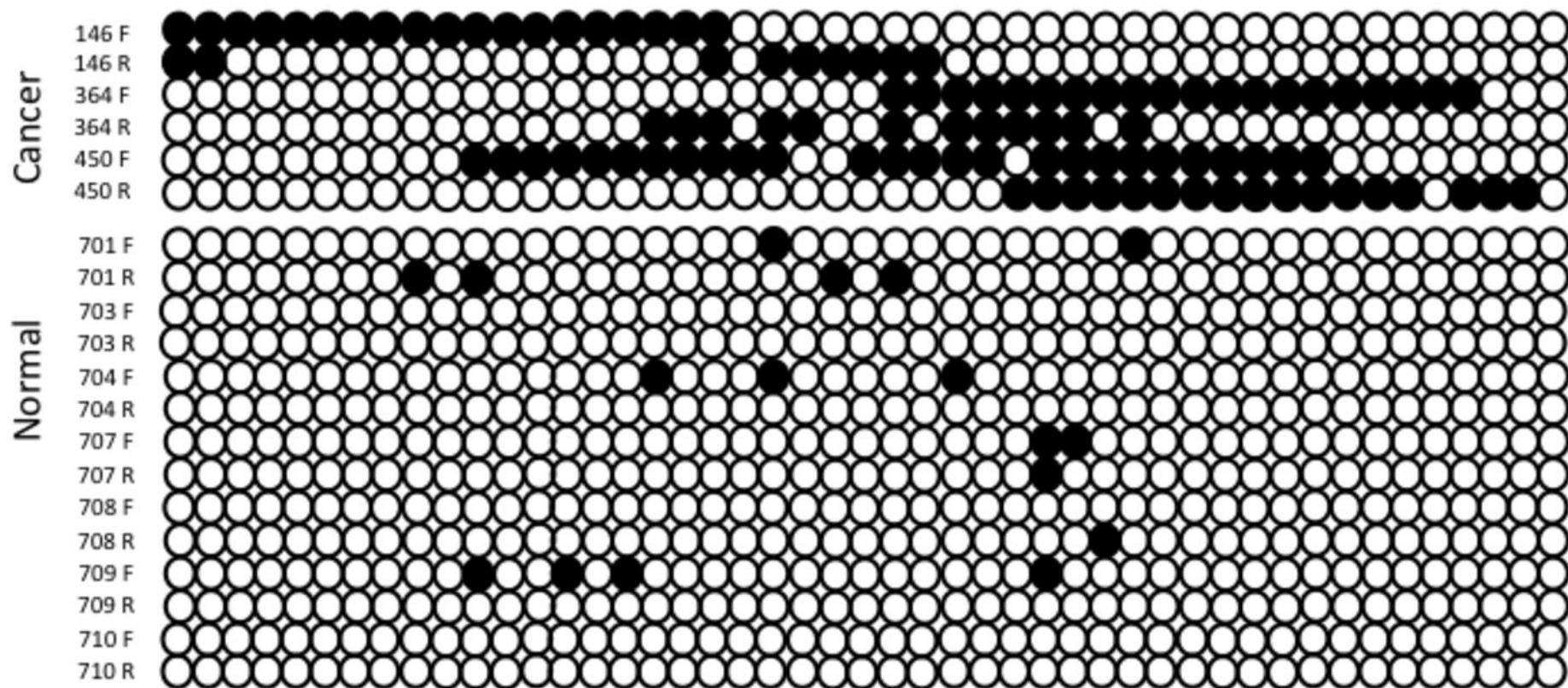
Cancer   Normal

A

Scale   500 bases| hg19
chr20: | 23,969,100   23,969,200   23,969,300   23,969,400   23,969,500   23,969,600   23,969,700   23,969,800   23,969,900
                                                              RefSeq Genes
GGTLC1

                                                    CpG Islands (Islands < 300 Bases are Light Green)
CpG: 25

B

A

Scale | 500 bases | hg19
chr7: 1,543,900 1,544,000 1,544,100 1,544,200 1,544,300 1,544,400 1,544,500 1,544,600 1,544,700 1,544,800

RefSeq Genes

INTS1

CpG Islands (Islands < 300 Bases are Light Green)

CpG: 119

B

Cancer
146 F
146 R
450 F
450 R

Normal
700 F
700 R
701 F
701 R
702 F
702 R
705 F
705 R
706 F
706 R
707 F
707 R
709 F
709 R

A

Scale | 500 bases |
chr18: | 74,206,800 | 74,206,900 | 74,207,000 | 74,207,100 | 74,207,200 | 74,207,300 | 74,207,400 | 74,207,500 | 74,207,600

RefSeq Genes
ZNF516
FLJ44313
CpG Islands (Islands < 300 Bases are Light Green)
CpG: 151

B

Cancer
44 F
44 R
57 F
57 R
64 F
64 R
146 F
146 R
364 F
364 R
450 F
450 R
481 F
481 R

Normal
700 F
700 R
701 F
701 R
702 F
702 R
703 F
703 R
705 F
705 R
706 F
706 R

Normal | Low Grade Lesions | High Grade Lesions | Cervical cancer

GGTLA4
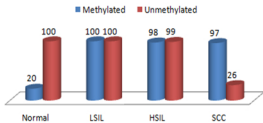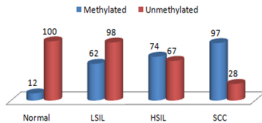
Methylated ■ Unmethylated

	Methylated	Unmethylated
Normal	40	96
LSIL	59	85
HSIL	97	90
SCC	100	10

ZNF516

Methylated ■ Unmethylated

	Methylated	Unmethylated
Normal	44	96
LSIL	100	95
HSIL	99	98
SCC	97	15

FKBP6

Methylated ■ Unmethylated

	Methylated	Unmethylated
Normal	12	100
LSIL	85	64
HSIL	65	67
SCC	100	18

INTS1

Methylated ■ Unmethylated

	Methylated	Unmethylated
Normal	20	100
LSIL	100	100
HSIL	98	99
SCC	97	26

SAP130

Methylated ■ Unmethylated

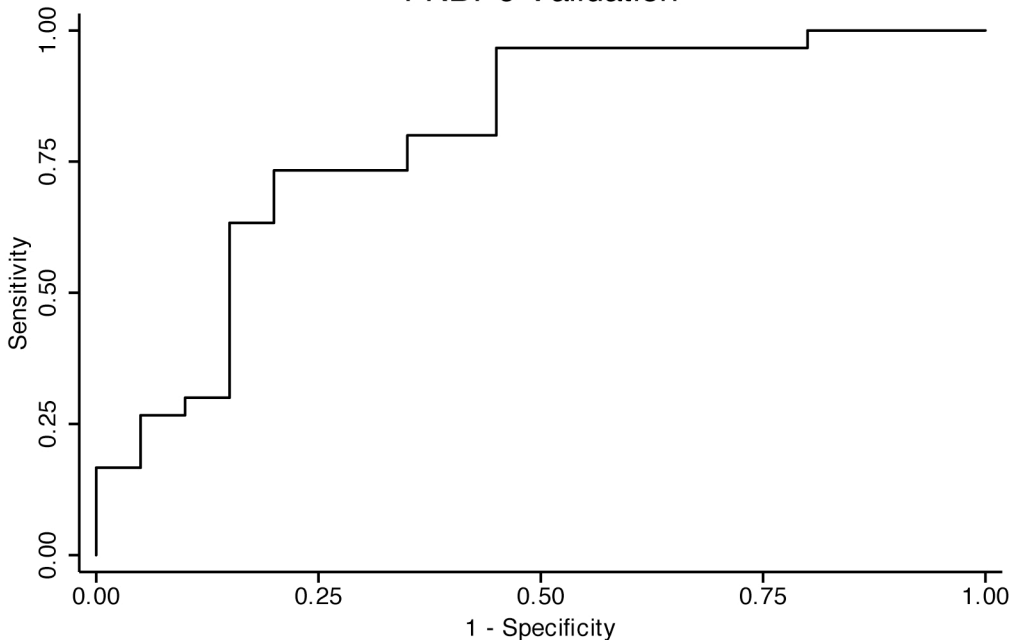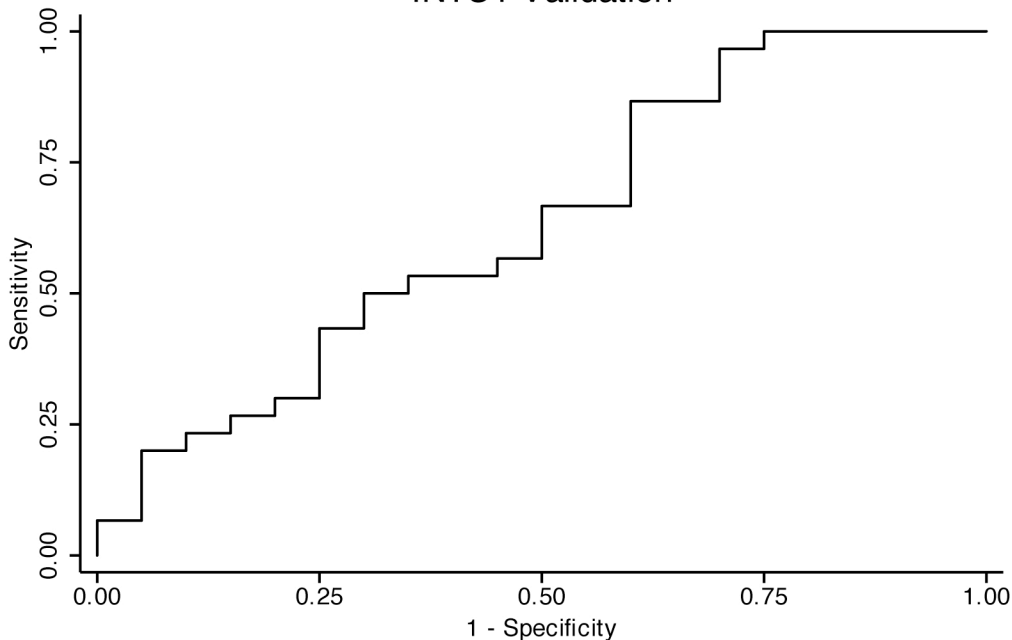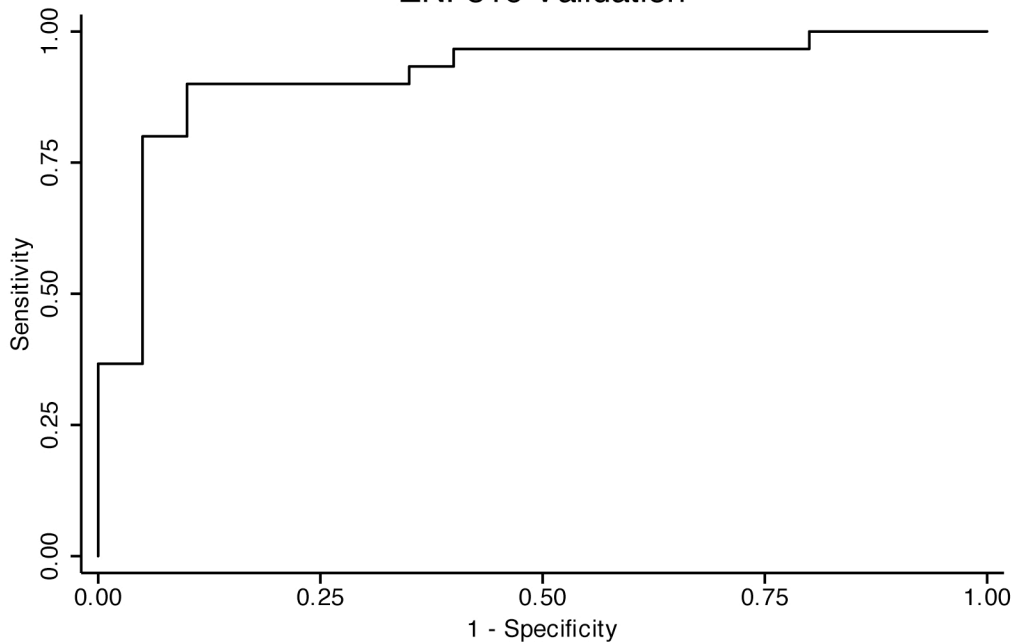|	Methylated	Unmethylated
Normal	12	100
LSIL	62	98
HSIL	74	67
SCC	97	28

FKBP6 Validation

Area under ROC curve = 0.80
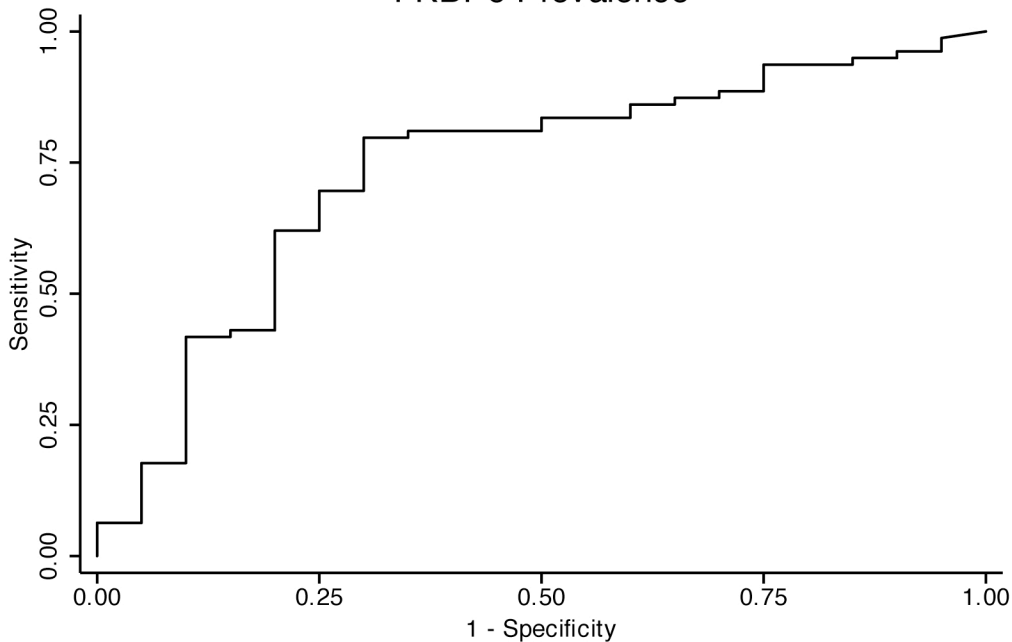
INTS1 Validation

Area under ROC curve = 0.63
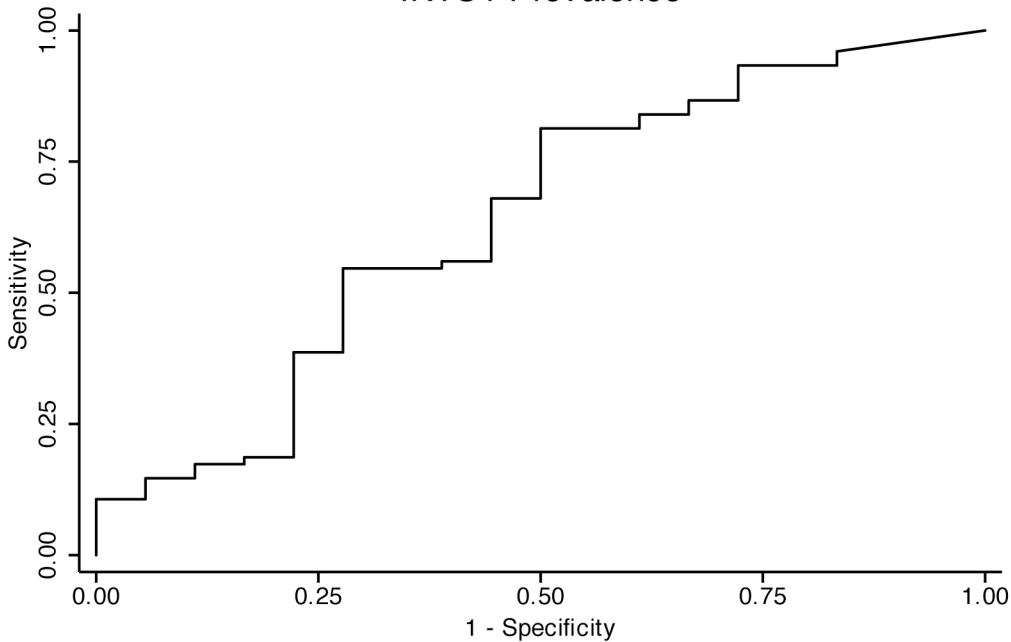
ZNF516 Validation

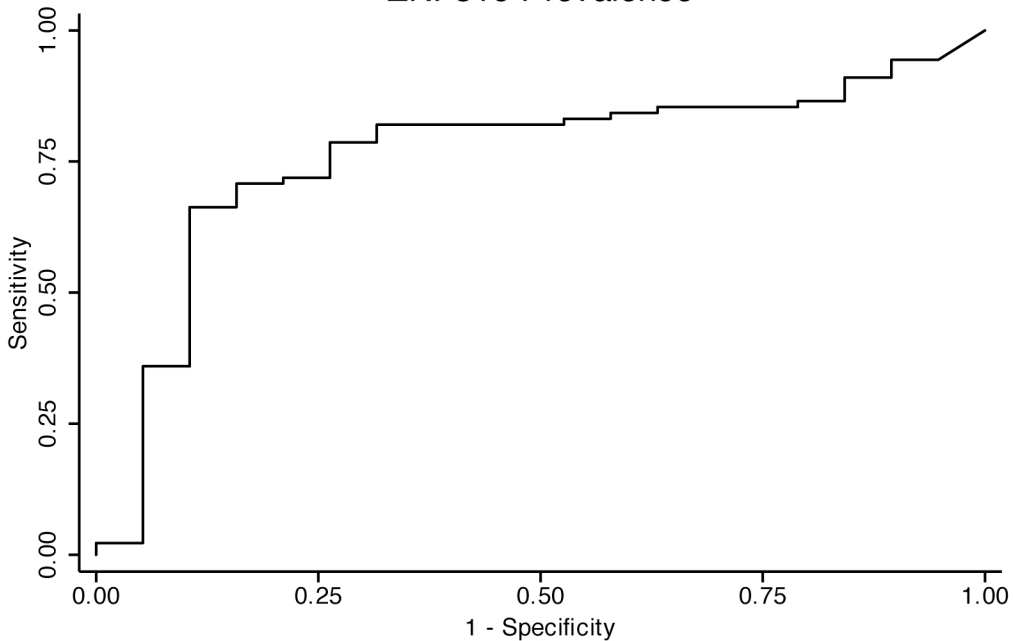Area under ROC curve = 0.92

FKBP6 Prevalence

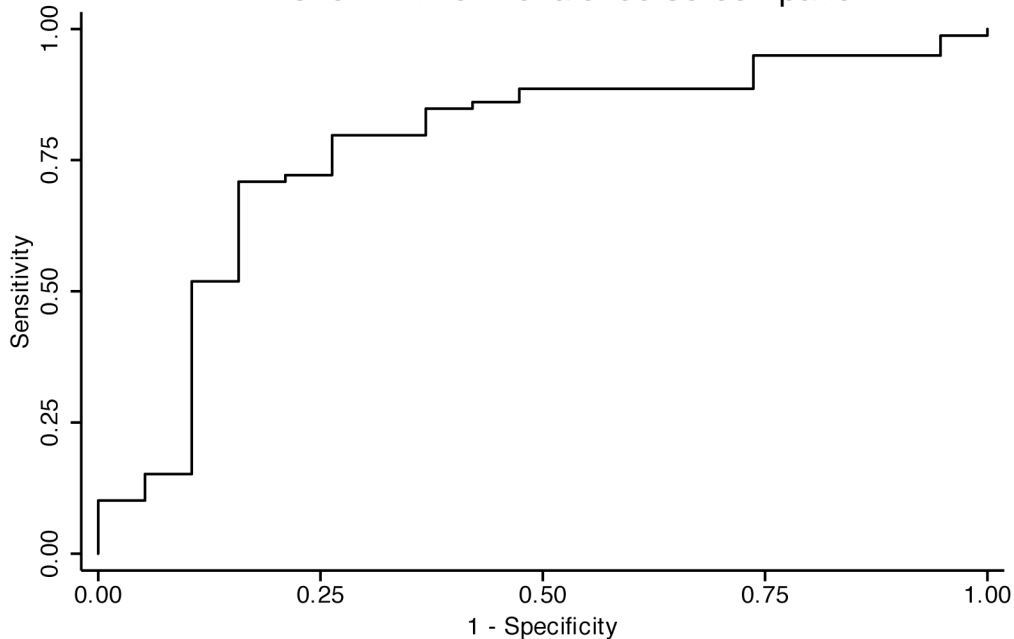Area under ROC curve = 0.74

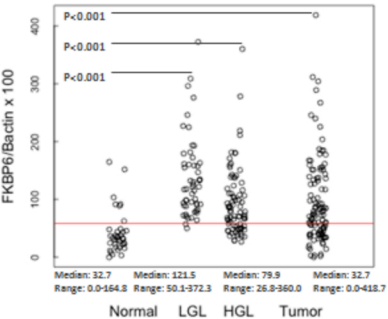INTS1 Prevalence

Area under ROC curve = 0.64

ZNF516 Prevalence

Area under ROC curve = 0.76

ZNF516 FKBP6 Prevalence screen panel

Area under ROC curve = 0.78

FKBP6

P<0.001
P<0.001
P<0.001

Median: 32.7
Range: 0.0-164.8

Median: 121.5
Range: 50.1-372.3

Median: 79.9
Range: 26.8-360.0

Median: 32.7
Range: 0.0-418.7

Normal LGL HGL Tumor

INTS1

P=0.012
P=0.045
P=0.005

Median: 40.3
Range: 0.0-147.6

Median: 55.8
Range: 18.0-157.7

Median: 48.1
Range: 19.0-194.1

Median: 55.0
Range: 0.0-513.3

Normal LGL HGL Tumor

ZNF516

P<0.001
P<0.001
P<0.001

Median: 84.9
Range: 0.0-209.3

Median: 235.9
Range: 74.3-773.7

Median: 136.4
Range: 31.6-1064.7

Median: 273.8
Range: 0.0-1220.7

Normal LGL HGL Tumor

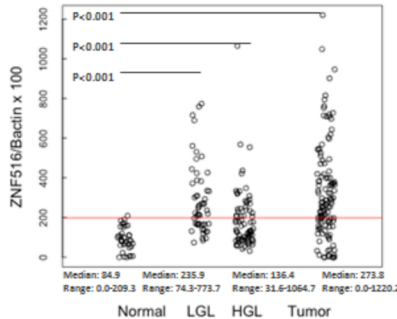**Supplementary Figures Legends**

Supplementary Figure 1a. Subset of statistically significant (p<0.01; q<0.05) methylated probes with more than a two-fold change differential methylation value when comparing normal to tumor samples ordered from the beginning of the p arm to the end of the q arm in odd numbered chromosomes from 13-19.

Supplementary Figure 1b: Subset of statistically significant (p<0.01; q<0.05) methylated probes with more than a two-fold change differential methylation value when comparing normal to tumor samples ordered from the beginning of the p arm to the end of the q arm in chromosomes 20, 21, 22 and in the X chromosome.

Supplementary Figure 2a-e. Representative bisulfite sequencing results for *FKBP6*, *GGTLA4, INTS1, SAP130*, and *ZNF516* for samples that were hybridized to the methylation arrays. Methylated (black dots) and unmethylated (white dots) cytosines in PCR fragments amplified with bisulfite sequencing primers. The promoter region around the TSS site of these five genes (A) and the graphical representations of the bisulfite sequencing results (B), tested in the Discovery cohort are shown in Supplementary Figures 2a) *FKBP6*; Fig. 2b) *GGTLA4*; Fig. 2c) *INTS1*; Fig. 2d *SAP130*; and Fig. 2e) *ZNF516*.

Supplementary Figure 3a). MSP results in the samples that were hybridized to the microarrays. M: Methylated, U: Unmethylated, C+ 100% Methylated Bisulfite treated DNA (Zymo Research), C- PCR product without DNA (blank). (708-710 Normals, 57-481 tumor)

Supplementary Figure 3b). Representative methylation specific PCR (MSP) results for A: B-actin (268 bp), B:GGTLA4 (M183, U185 bp), C: FKBP6 (M137, U135 bp), D: ZNF516 (M 241, U 242 bp), E: INTS1 (M143, U147 bp) and F: SAP130 (M189, U192 bp) by histology type in normal, LSIL, HSIL and cervical cancer samples. M: Methylated, U: Unmethylated.

Supplementary Figure 3c). Bar charts of percentage of methylation by MSP across normal (n=25), LSIL (n=66), HSIL (n=91), and cervical SCC (n=39) samples. Methylated is presence of a band in the methylated MSP. Unmethylated is presence of a band in the unmethylated MSP. In the normal samples the genes FKBP6 (12%), INTS1 (20%) and, SAP130 (12%) showed less methylation that GGTLA4 and ZNF516 (40% and 44% respectively). In the LSIL the frequency of methylation increases for all the genes, GGTLA4 (59%), FKBP6 (85%), ZNF516 (100%), INTS1 (100%) and SAP130 (62%). In the HSIL the methylation frequency varied. Only GGTLA4, and SAP130 have increases in the rates of methylation (97% and 74% respectively), while FKBP6 (65%), ZNF516 (99%) and INTS1 (98%) decreased. Finally, in the cancer samples, the genes GGTLA4, FKBP6 and SAP130 show increases in methylation frequency from 97% to 100%. For GGTLA4, and SAP130 the methylation frequency increases with the severity of the cervical lesion.

Supplementary Figure 4a-g. Receiver Operator Characteristics (ROC) curves and Area Under the Curve (AUC) results in the Validation cohort for Fig. 4a) *FKBP6;* Fig. 4b) *INTS1*; and Fig. 4c) *ZNF516.* ROC curves and AUC results in the Prevalence cohort for Fig. 4d) *FKBP6;* Fig. 4e) *INTS1*; and Fig. 4f) *ZNF516.* ROC curves and AUC results in the Validation and Prevalence cohorts for Fig. 4g) a combined panel of *FKBP6* and *ZNF516.*

Supplementary Figure 5. Scatterplots of qMSP analysis of *FKBP6, INTS1*, and *ZNF516* in all normal (n=37), LSIL (n=53), HSIL (n=84), and cancer (n=120) samples. The relative level of methylated DNA for each gene in each sample was determined as a ratio of qMSP for the amplified gene to β-actin. Red line denotes cut-off value.