Supplementary information

DNA methylation epigenotype and clinical features of NRAS-mutation(+) colorectal cancer

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Supplementary Tables:

Supplementary Figures:

Supplementary Table S1. Methylation marker genes and primer sequences for pyrosequencing

Gene	PCR Primers (Forward/Reverse)		Sequencing primers
Group-1			
SPON1	**F**	AGGAGGTTGTTGTTTTAATTTTTAGTTATA	AGTTATATTTAGGATTTTTTGGAG
	R*	AATTATTTCTCAAACTTTCCCTCCTCTA	
TIMP3	**F**	AGAGATATTTAGTGGTTTAGGTG	GGTGGGAGTGGGGTTA
	R*	AAACCCTCCTACCCCTTCT	
CACNA1G	**F**	TGTTTTGGTTTAAGTAGAAGAAAAT	TGGTTTAAGTAGAAGAAAATTAT
	R*	ACCCAAATCAACAAAAAAAAACCC	
MLH1	**F**	ATGTGGATGAAGTTTAAAAGAAGTAAGAT	ATGGAAGTAGAAGAGGTTTTAGTTT
	R*	AAAACTCCACCACCAAATA	
MINT17	**F***	AAGTGGGAGAAGAGGAAGAGAAAAAATA	TATCCCTCCCCATCT
	R	CTATCCTCCCCAAACTTCT	
p16INKA	**F***	GGTGGGTAGAGGGTTTGTA	GGGAGTAGGGGATGG
	R	CCAATTCCCCTACAAACTT	
Group-2			
ADAMTS1	**F**	GTTTTTTGGGGTTTTAATGTAG	TTAATGTAGAGAGTTGTGTT
	R*	TTCCCCATATCCCTACCCAACTTAC	
BNIP3	**F**	AGAAGTAGATTTATTTTTAGGTGGAATT	ATTTTTAGGTGGAATTTTAGT
	R*	AAAAAACCCCCATTCTCCAACT	
EDIL3	**F**	GGGATTTTTAGTTTATTTTTTATTTAGTTG	TTAGTTTATTTTTTATTTAGTTGTT
	R*	ACTCAACCTCCATATCCCCCAATT	
EFEMP1	**F***	TTGGGAAGTTGAGTAGTTTTAGGG	AAATCCCCTTTCTTAACA
	R	ACCCCACAAAAAAATAAAATCCCCTTTCTT	
ELMO1	**F**	GGAGGAAGGAAGAGGAAGTGA	GAGGAAGTGAGAGTAG
	R*	CCAACACACCCACTTACACTCTAAAT	
PPP1R3C	**F**	GTGTTTGGGAGTAGATAAG	TTTTTAGGAGTAGGGTTTTTAGTTT
	R*	CACAACTCCAAACCTTACC	
PPP1R14A	**F**	GGGGGTTTGGGATAGATAT	GGGATTAGGTTTGTTTG
	R*	CTCCCCCACCAACAACCC	
RASSF2	**F**	AGGGTTGTTAGGTTTTTTTTAGT	GTTAGGTTTTTTTTAGTTTTGTA
	R*	CCCATCCCCCAAATCTCTAAAACTT	
STOX2	**F**	AGGTTGGGGTAGTTGTTAAG	GGGTAGTTGTTAAGGTTT
	R*	TCCCATCAAACTTCTCATTTTCA	
TMEFF2	**F**	ATTTAGGGATTGGGTTTAGT	AGGGTAGTTAGTTGAGAAGT
	R*	CCCTCCTTATAACAACAACT	
UCHL1	**F***	GGTAGGGTTTTTAAATTTTGTAGTTTTATT	CCACCAAATTATCTCACC
	R	CCACAACCACCAAATTATCT	
ZNF447	**F**	GGGGTAGTTGAGTAGTAGGTGG	GGTAGGTTTAGGGGATGTAG
	R*	CCTCACCCTCTACCCTATTAAAATC	
NEUROG1	**F**	AGTTTGGGGTTGTTATTTTGTGTTA	GTTGTTATTTTGTGTTAGTTG
	R*	AAAAAACCAAACCAAATTCTCC	

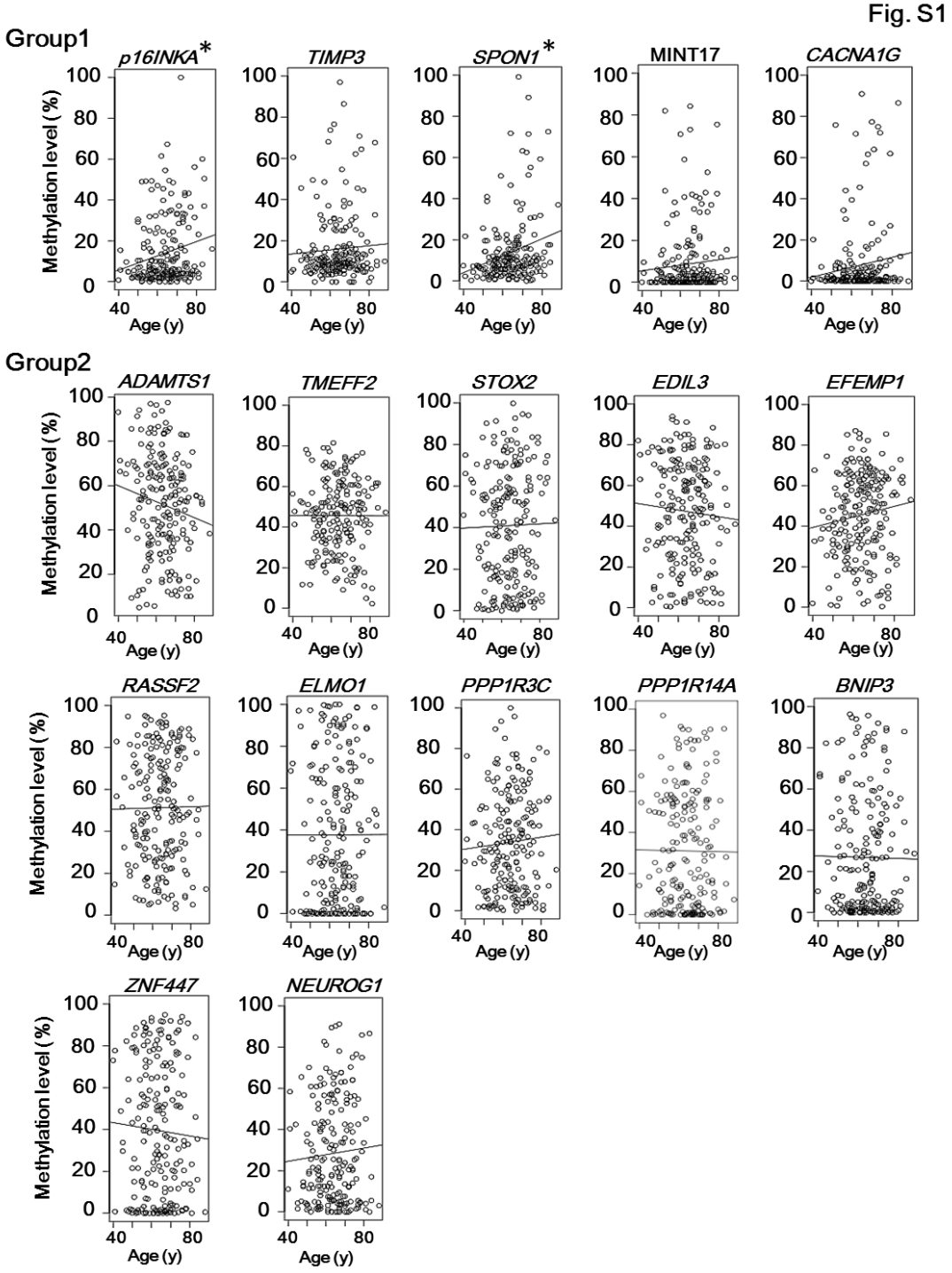
* Primters with 5´-biotin tag.

Supplementary Table S2. Comparison of clinicopathological features of all CRC cases

Clinical features		All cases	*BRAF*	*KRAS*	*NRAS*	No-mut	*P*-value (*K* vs *N* vs *No*)	*P*-value (*K* vs *N*)
# of sample		205	13	59	56	72		
Gender								
	Male	115	9	33	25	48	0.2	0.6
	Female	84	4	26	25	24		
	Unkown	6	0	0	6	0		
Age (y.o.)								
	Mean±SD	63.8±9.4	71.0±8.5	61.7±9.4	66.7±9.1	62.0±9.1	*0.005	*0.01
Tumor location								
	Proximal	61	13	23	6	17	$*2\times10^{-5}$	*0.002
	Distal	137	0	36	43	55		
	Unkown	7	0	0	7	0		
Mucinous component								
	(+)	31	8	14	3	5	*0.004	*0.02
	(-)	167	5	45	46	67		
	Unkown	7	0	0	7	0		
AJCC Stage								
	I	15	3	0	11	0	$*5\times10^{-6}$	*0.001
	II	54	5	17	16	16		
	III	64	3	21	11	26		
	IV	66	2	21	12	30		
	Unkown	6	0	0	6	0		
Lymph node metastasis								
	(+)	108	5	39	17	43	*0.005	*0.003
	(-)	87	8	20	30	28		
	Unkown	10	0	0	9	1		
Lymph vessel invasion								
	(+)	135	11	48	19	53	$*5\times10^{-6}$	$*8\times10^{-6}$
	(-)	63	2	11	30	19		
	Unkown	7	0	0	7	0		
Venous invasion								
	(+)	157	9	48	33	62	*0.04	0.1
	(-)	41	4	11	16	10		
	Unkown	7	0	0	7	0		
Microsatellite instability								
	MSI-H	17	11	3	0	3	0.3	0.3
	MSS	180	2	56	48	69		
	Unkown	8	0	0	8	0		
Methylation epigenotype								
	HME	13	9	2	0	2	$*3\times10^{-4}$	$*1\times10^{-4}$
	IME	89	4	40	18	27		
	LME	98	0	17	38	43		

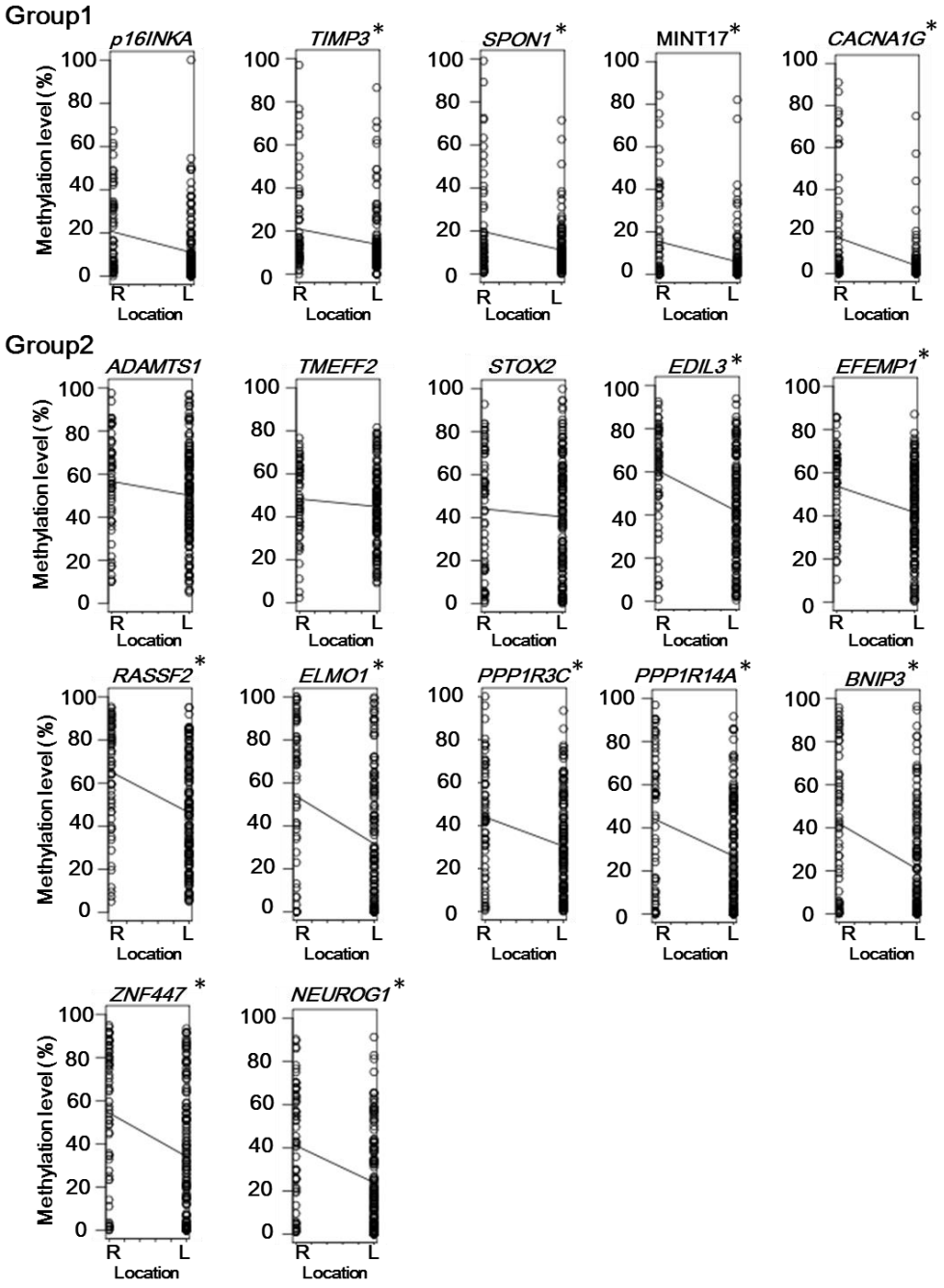
No-mut: no mutation. *B vs K vs N*: *BRAF* vs *KRAS* vs *NRAS*. *K vs N*:*KRAS* vs *NRAS*. *MSI-H*: microsatellite instability high. *MSS*: microsatellite stable. *$P < 0.05$

Fig. S1



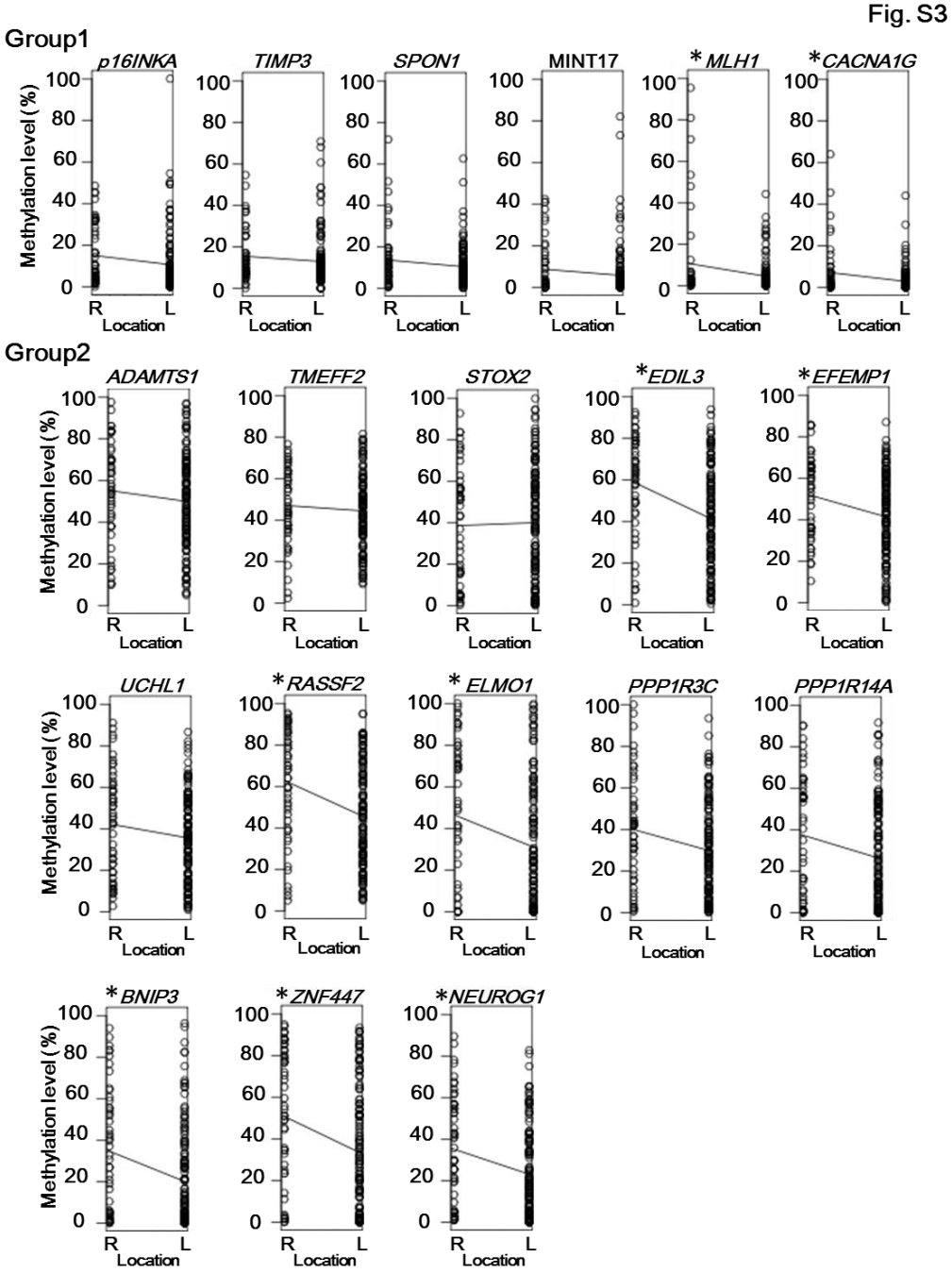
Supplementary Figure S1. Comparison between methylation levels and age using linear single regression model. Three of the six Group-1 markers showed a significant correlation between higher methylation levels and age (*P < 0.008), while none of Group-2 markers did. Since six Group-1 markers were evaluated, P-values <0.008 (i.e., 0.05/6) were considered significant.

Fig. S2



Supplementary Figure S2. Comparison between methylation levels and tumor location using a linear single regression model. While five of the Group-1 markers showed a significant correlation between higher methylation level and proximal location (*P < 0.008), 10 of 13 Group-2 markers showed significant correlation between higher methylation level and proximal location (*P < 0.004). Since six Group-1 markers and 13 Group-2 markers were evaluated, P-values <0.008 (i.e., 0.05/6) and <0.004 (i.e., 0.05/13), respectively, were considered significant.

Fig. S3



Supplementary Figure S3. Comparison between methylation levels and tumor location using a linear single regression model, excluding HME CRCs. While two of the Group-1 markers showed a significant correlation between higher methylation level and proximal location (*P < 0.008), 7 of 13 Group-2 markers showed a significant correlation between higher methylation level and proximal location (*P < 0.004). Since six Group-1 markers and 13 Group-2 markers were evaluated, P-values <0.008 (i.e., 0.05/6) and <0.004 (i.e., 0.05/13), respectively, were considered significant.