Figure 1. Summary of chromosomal aberrations and their frequencies in 39 CRC specimens determined using array-CGH analysis. Losses (green bars) are displayed on the left, and gains (red bars) are on the right. The chromosome ideogram was generated using Genomic Workbench software.

BRAF mutation and MSI are significantly more prevalent among CIMP-high (CIMP-H or CIMP1) CRCs, whereas *KRAS* mutation is more prevalent among CIMP-low (CIMP-L or CIMP2) tumors (Ogino et al., 2006; Shen et al., 2007b; Hinoue et al., 2012).

Inverse Correlation Between DNA Methylation and Chromosomal Alterations

To quantitatively evaluate copy number aberrations on a genome-wide scale, we calculated the total numbers and lengths of CNAs (losses + gains) identified by the array-CGH analysis. We observed a strong correlation between the total numbers of CNAs in the CRC samples tested and the total lengths of the CNAs (Supporting Information Fig. 2). We therefore used the total CNA length as an index representing the degree of chromosomal alteration and assessed the relationship between total CNA length and methylation status. We found that total CNA lengths were smaller in CRCs with frequent DNA methylation than in those without frequent methylation ($P = 0.033$, Fig. 2C). Interestingly, when we analyzed genomic losses and gains separately, we again observed a significant difference in the magnitude of the losses between frequent methylation-positive and -negative tumors, whereas no such difference was found for gains (Figs. 2A and 2B). We also analyzed the relationship between CNAs and mutation of *TP53* or *KRAS* but found no statistically significant correlations (Supporting Information Fig. 3).

Chromosomal Alterations and Their Association with Clinical Stage in CRC

To determine whether chromosomal alterations accumulate during the progression of CRCs, we assessed the CNA status of tumors at each Dukes' stage. When genomic gains and losses were analyzed separately, we found a tendency toward greater genomic losses in tumors at higher Dukes' stages, but the trend was not statistically significant (Fig. 3A). By contrast, Dukes' C tumors showed the greatest genomic gains, whereas Dukes' D tumors exhibited unexpectedly small gains (Fig. 3B). The total CNA lengths were greatest in Dukes' C tumors, and again Dukes' D tumors showed less chromosomal alteration than Dukes' C tumors (Fig. 3C).

We found that losses at several loci, including 3p24.3, 4p13-15.31, 5qcen-11.2, 8p11-q11, 9p21.3-21.1, 17q24.2-24.3, and 22q13.31, were prevalent in tumors with distant metastasis (Dukes' D) (Supporting Information Table 2), while gains at 11q13.1-13.2, 17q12, and 17q21.2 were prevalent among Dukes' D tumors (Supporting Information Table 3). In addition, losses at 4q21-34, 5q12.1, 5q35.3, and 11qcen-12.1 and gains at 2p15-16.1, 2p13.3, 5p13, 5p35.3, 6p21, 8q12.1-12.3, and 19q13.31 were commonly observed in tumors with lymph node metastasis (Supporting Information Tables 4, 5).

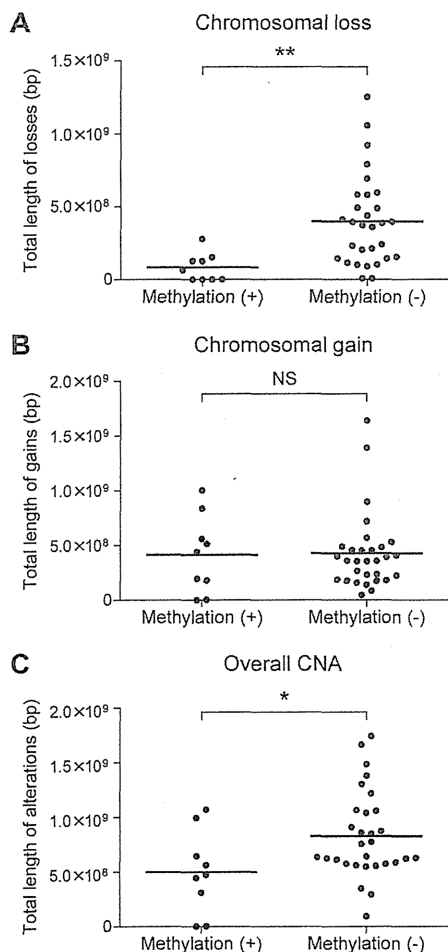


Figure 2. Association between CNAs and DNA methylation status. Total lengths of genomic losses (A) and gains (B) and overall CNAs (C) in tumors with and without frequent DNA methylation are shown. Each dot represents a single tumor. Methylation (+), tumors with frequent DNA methylation; Methylation (−), tumors without frequent DNA methylation; *$P < 0.05$; **$P < 0.01$; NS, not significant.

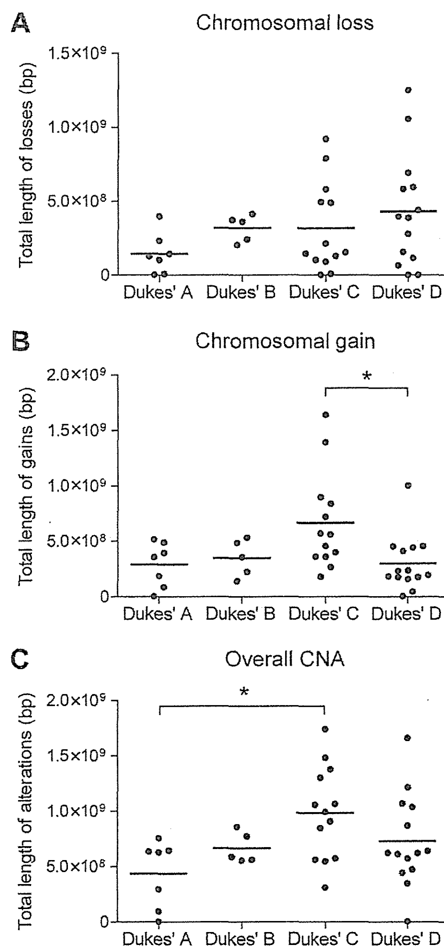


Figure 3. Association between CNAs and Dukes' stages. Total lengths of genomic losses (A), gains (B), and overall CNAs (C) in CRCs at each Dukes' stage are shown. *$P < 0.05$.

Clustering Analysis of CNAs and Their Association with Clinical Stage in CRC

Previous studies have shown that categorization of CRCs according to their chromosomal aberrations has strong relevance to their clinical behavior (Hermsen et al., 2002; Postma et al., 2009; Poulogiannis et al., 2010). For that reason, we carried out unsupervised clustering analysis using our array-CGH data (excluding the sex chromosomes) and then compared the results with genetic mutations and epigenetic alterations (Fig. 4A). We found that CRCs could be subcategorized into at least four clusters based on their CNAs. Gene mutations, DNA methylation status, and genomic alterations on representative chromosomes in each cluster are summarized in Table 2.

Tumors in cluster 1 are characterized by infrequent genomic losses and gains (Figs. 4A–4C). Losses were most prevalent among tumors in cluster 2, while gains were most prevalent among tumors in cluster 4 (Figs. 4A–4C). The total CNA lengths were greater in tumors in clusters 2 and 4 than in clusters 1 and 3 (Fig. 4D). Tumors with frequent DNA methylation were most strongly enriched in cluster 1 (5 of 9, 56%). Tumors in cluster 1 were also characterized by frequent *KRAS* mutation (6 of 10, 60%) and infrequent *p53* mutation (3 of 10, 30%), whereas *p53* mutation was most prevalent in cluster 3 tumors (10 of 15, 67%), although the difference was not statistically significant. Importantly, Dukes' D tumors were highly enriched in cluster 2, within which tumors showed the greatest genomic losses. By contrast, Dukes' C tumors were enriched in cluster 4 and

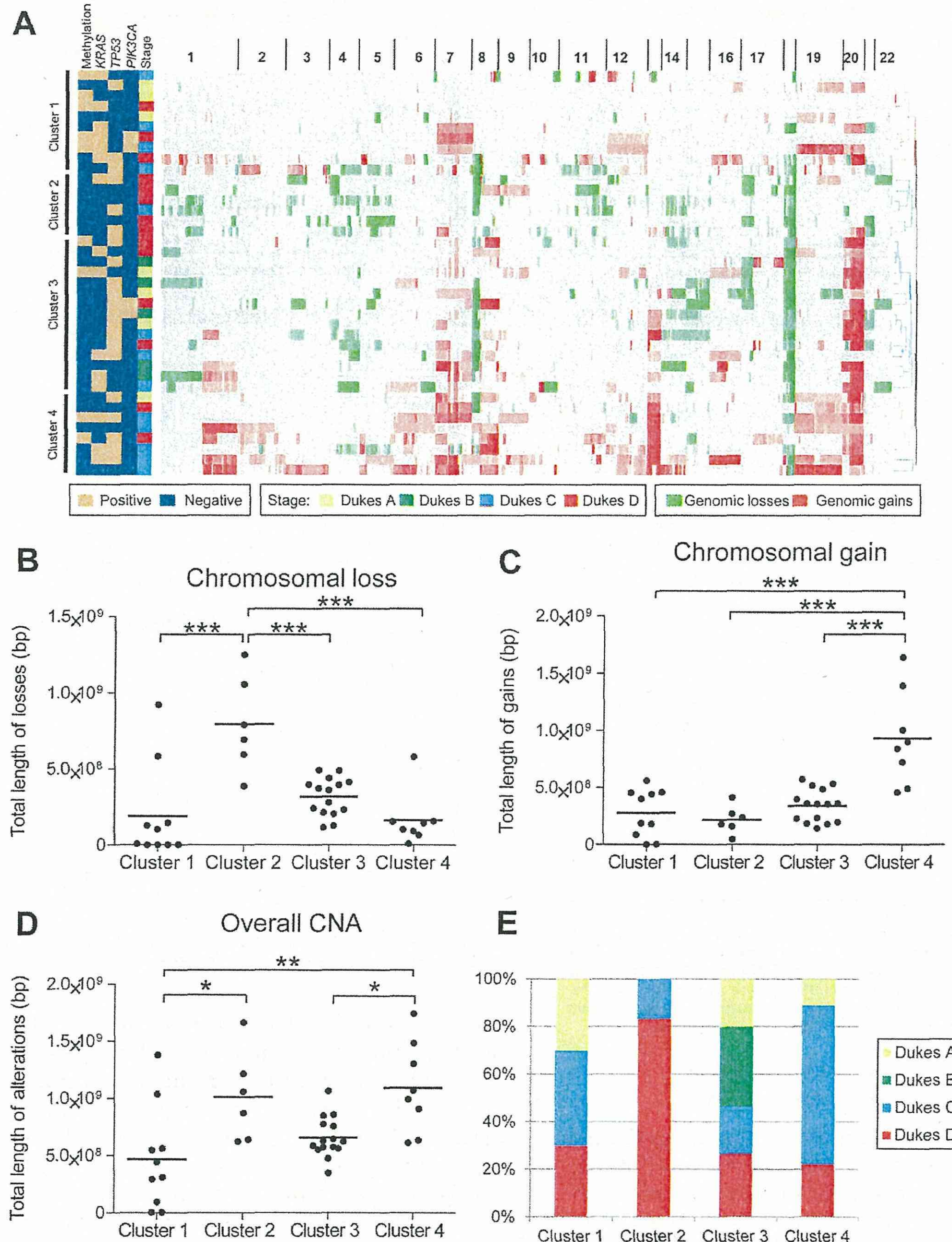


Figure 4. (A) Summarized results of unsupervised hierarchical analysis using the array-CGH data obtained from the 39 CRCs. Tumors were categorized into four clusters as indicated on the left. Tumors with frequent DNA methylation, *KRAS* mutation, *TP53* mutation, *PIK3CA* mutation, and Dukes' stages are also indicated on the left. (B–D) Total lengths of genomic losses and gains and overall CNAs in tumors within each cluster are shown. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (E) Percentages of tumors at the respective Dukes' stages in each cluster are shown.

showed the greatest genomic gains. These results suggest that there is an inverse relationship between CIN and aberrant DNA methylation, and that there is a possible association between genomic losses and distant metastasis and between genomic gains and lymph node metastasis in CRC.

To determine whether a similar clustering pattern could be seen in an independent set of CRC samples, we carried out the same unsupervised

TABLE 2. Mean DNA Copy Number Alterations Within the Four Groups Obtained from the Unsupervised Clustering Analysis

Cluster	Cluster 1	Cluster 2	Cluster 3	Cluster 4	*P* value
Sample	10	6	15	8	
Age (mean ± SD)	66±14.7	69±11.2	69±10.1	63±15.2	
Sex					
Male	4	3	10	7	
Female	6	3	5	1	
Methylation	5/10*	0/6	2/15	2/8	
KRAS	6/10	1/6	5/15	3/8	
TP53	3/10	3/6	10/15	5/8	
PIK3CA	2/10	0/6	2/15	0/8	
18q loss	4/10*	6/6	15/15*	5/8	
20q gain	4/10*	3/6	14/15*	8/8	
18p loss	3/10*	4/6	13/15*	3/8	
8p loss	2/10*	4/6	11/15	4/8	
13q gain	1/10*	2/6	8/15	8/8*	
20p gain	2/10	0/6*	9/15	6/8*	
7p gain	4/10	1/6	5/15	6/8*	
7q gain	4/10	0/6*	7/15	5/8	
Loss (Mb)	189.4	795.8	318.5	162.6	<0.001
Gain (Mb)	277.4	216.3	339.1	930.3	0.001
Total CNA (Mb)	466.8	1012.1	657.6	1092.9	<0.001

Figures are the mean area of the CNAs per genome in each group, which were compared using one-way ANOVA. The total CNA is the sum of the losses and gains. Methylation, tumors with frequent DNA methylation.
*Significantly different from a random distribution, determined from the absolute value of the adjusted standardized residuals >1.96.

hierarchical clustering analysis using the publicly available data set (n = 121) reported by Nakao et al. (2004). We found that the CRC samples were subcategorized into four clusters, among which group 1 showed the highest levels of copy number gains, while group 3 showed the greatest genomic losses (Supporting Information Fig. 4).

DISCUSSION

CIN, which is inferred from DNA ploidy patterns, is the most common form of genomic instability in CRC and is defined by the presence of multiple structural or numerical chromosome changes (Rajagopalan and Lengauer, 2004; Grady and Carethers, 2008). Because mutations in mitotic checkpoint regulators are found in only a small population of CIN CRCs, the mechanism underlying CIN is largely unknown (Grady and Carethers, 2008). Moreover, no method for accurately evaluating the extent of CIN has yet been established. In this study, we used the total CNA length as a surrogate for CIN and analyzed its association with clinical and molecular variables. As described in an earlier study (Gaasenbeek et al., 2006), losses of heterozygosity (LOH) without accompanying copy number changes (e.g., somatic uniparental disomies) were not included as CNAs due to CGH array limitations. It is well established that estimates of CIN prevalence and mutation detection are influenced by the method used for analysis and the purity of the samples (Nakamura et al., 1994; Habano et al., 1996; Sugai et al., 2000; Cardoso et al., 2004; Issa, 2008). We therefore used a crypt isolation technique to avoid contamination by nontumorous cells. The high levels of marker gene methylation detected by quantitative pyrosequencing reflect the purity of the cancer cells in the isolated gland specimens and are indicative of the advantage of using the crypt isolation technique.

Several groups have reported an inverse relationship between CIMP and CIN (Goel et al., 2007; Cheng et al., 2008). Goel et al. (2007) found that CIN status measured as the LOH for eight microsatellite markers was inversely correlated with the methylation frequency at CIMP-related markers in sporadic CRCs, even those without MSI. In addition, Cheng et al. (2008) found that CIN measured as the number of chromosomal arms with gains/losses or LOH was inversely associated with CIMP status. In this study, we also observed an inverse relationship between concurrent methylation at multiple loci (which may represent CIMP) and CIN, although none of the tumors in this study exhibited *MLH1* methylation. Interestingly, we detected no significant difference in the magnitude of the chromosomal gains between tumors with frequent methylation and those without it. This suggests gains at certain genomic loci may be commonly involved in the pathogenesis of CRCs, irrespective of the methylation status. The presence of tumors with a high degree of chromosomal aberration in a subset of CIMP tumors may support this idea (Cheng et al., 2008).

There have been few studies in which the extent of chromosomal aberration during the progression of CRCs was analyzed as we have done in the present study. Hermsen et al. (2002) used conventional CGH to show that the progression of adenoma to carcinoma is associated with an increase in chromosomal aberration. A meta-analysis of 31 conventional CGH studies also showed that primary metastatic CRCs had significantly greater genomic alterations than non-metastatic CRCs (Diep et al., 2006). In addition, the results

of our array-CGH analysis suggest that accumulation of chromosomal losses may play an important role in the progression of CRCs from Dukes' A to Dukes' D. Unexpectedly, however, the overall level of CNAs was highest in Dukes' C tumors, with Dukes' D tumors showing somewhat less chromosomal alteration, which is probably attributable to the greater genomic gains in the Dukes' C group. Assuming that chromosomal gains are irreversible during CRC progression, at least a subset of Dukes' D tumors may have originated as Dukes' A or B tumors, not Dukes' C tumors with their larger chromosomal gains. Our results thus suggest that genomic losses may have a more significant impact on the metastatic properties of tumor cells than genomic gains, and that tumor cells that acquire losses at early stages in regions that are critical for metastasis may have a greater chance to metastasize to distant organs.

We also assessed the association between CNAs and tumor progression, including lymph node and distant metastasis. A previous meta-analysis of conventional CGH data suggests that losses at 4p are associated with the progression from Dukes' A tumors to Dukes' B–D tumors, and losses at 8p and gains at 7p and 17q correlate with liver metastasis (Diep et al., 2006). Our present findings that Dukes' D tumors frequently show losses at 4p and 8p and gains at 17q are consistent with those earlier findings. Importantly, our results seem to reflect the recent findings that losses at 4p and 8p are indicators of liver metastasis and a poor prognosis in CRC (Sheffer et al., 2009).

From an epigenetic viewpoint, Ju et al. (2011) reported that larger numbers of genes were methylated in stage I-III CRCs than in stage IV samples, and that CRCs at stages I-III exhibited methylation profiles that distinctly differed from the profiles of stage IV tumors. Together with the observation that even early stage tumors show intratumor heterogeneities, including ploidy pattern variation (Miyazaki et al., 1999), allelic imbalances (Boland et al., 1995; Sugai et al., 2005), and gene mutations (Baldus et al., 2010), tumor cells with metastatic potential may arise through early genetic and epigenetic lesions, as proposed in the "initiation" (Threadgill, 2005) or "parallel evolution" models (Gray, 2003).

In CRCs with distant metastasis, we frequently observed losses at 3q13.11, where *ACVR2B*, encoding activin A receptor type IIB, is located. Activins are growth and differentiation factors; they belong to the TGF-β superfamily and regulate cell differentiation, proliferation, and apoptosis in a variety of cancer cell types (Chen et al., 2006). Mutation of *ACVR2A*, which is 69% identical to *ACVR2B*, results in loss of its expression and is frequently found in MSI-positive CRCs (Jung et al., 2004). Furthermore, restoration of *ACVR2A* suppresses CRC cell growth, suggesting that it acts as a tumor suppressor (Jung et al., 2007). Together with an earlier report that expression of ACVR2B is weaker in CRC tissues than in normal colon mucosa (Babel et al., 2009), our findings suggest genomic loss of the *ACVR2B* locus may be involved in carcinogenesis and distant metastases in a subset of CRCs.

Consistent with the aforementioned meta-analysis of CGH data (Diep et al., 2006), we commonly observed genomic gains at 17q12, which encompasses a region encoding *ERBB2* and is frequently amplified and/or overexpressed in various types of cancer. One recent study showed that amplification of *ERBB2* leads to persistent activation of extracellular signal-regulated kinase 1/2 (ERK1/2) signaling, which in turn leads to resistance to treatment with cetuximab (Yonesaka et al., 2011). It has also been reported that the median overall survival was significantly longer for CRC patients without *ERBB2* amplification than for those with it (Yonesaka et al., 2011). Thus assessment of chromosomal aberrations at functionally important loci in CRCs could provide useful information for predicting distant metastasis and prognosis, as well as for the development of therapeutic strategies.

There have been a number of studies evaluating the utility of genomic instabilities, including CIN and CNAs, as prognostic markers in CRC (Pritchard and Grady, 2011). A recent meta-analysis confirmed that CIN (measured flow cytometrically as the presence of aneuploidy/polyploidy) is associated with an unfavorable prognosis (Walther et al., 2008). Our array-CGH analysis revealed an increase in copy number losses during the progression of tumor stage, suggesting that the total magnitude of genomic losses could potentially serve as a surrogate marker for genomic instabilities and a prognostic marker in CRC. Recently, Poulogiannis et al. (2010) carried out a hierarchical clustering analysis of array-CGH data obtained from 109 primary CRCs and showed that tumors could be categorized into four groups, in which tumors in groups I and II exhibited CNAs only infrequently, while those in groups III and IV exhibited an abundance of CNAs. CRCs in group I were characterized by a lack of CNAs and frequent MSI-positivity. This appears to be consistent with cluster 1 in our study, which was

enriched in tumors with frequent DNA methylation, and may suggest that the copy number profiles of CRCs with MSI are similar to those with frequent methylation but without *BRAF* mutation. They also reported that CRCs in group II, in which CNAs were somewhat more prevalent than in group I, were associated with a lack of lymph-node metastasis and a better prognosis, irrespective of MSI status (Poulogiannis et al., 2010). Thus group II may correspond to cluster 3 in our study, in which Dukes' B (lymph-node negative) tumors were significantly enriched. By contrast, most patients in our cluster 2 were staged as Dukes' D, with significantly greater genomic losses than tumors in other clusters. Furthermore, Dukes' C tumors were enriched in cluster 4, within which tumors exhibited the greatest genomic gains. Although the proportions of Dukes' C and D tumors in the study by Poulogiannis et al. differ from those in our study, the highest number of Dukes' D tumors were seen in group III and were characterized by significant genomic losses, while the majority of the Dukes' C tumors were in group IV and exhibited significant genomic gains. Although this distribution did not reach statistical significance, the tendency is consistent with our results. These findings suggest that cancers with gains are likely to metastasize to lymph nodes or unlikely to metastasize to distant sites. Thus a tumor's CNA pattern may define its metastatic behavior.

This study has several limitations, including a small sample size, the absence of tumors with *BRAF* mutation and/or *MLH1* methylation, and a lack of survival information; nonetheless, our results indicate several important findings. First, there is an inverse relationship between methylation status and the extent of the CNAs, particularly chromosomal losses. Second, tumor progression from Dukes' A to Dukes' C may be associated with the accumulation of CNAs, whereas a subset of Dukes' D tumors with smaller chromosomal gains may have been derived from Dukes' A or B tumors, but not Dukes' C tumors, which show significant genomic gains. Third, the different CNA patterns revealed by our hierarchical clustering analysis may be associated with different tumor behaviors, including local and distant metastasis, which suggests the presence of distinct molecular pathways in the development of CRC. Further study to clarify the differences between these subclasses will likely provide new insight into the molecular mechanisms that determine prognosis and the responses of CRCs to therapy.

ACKNOWLEDGMENTS

The authors thank Dr. William F. Goldman for editing the manuscript.

REFERENCES

Arai T, Kino I. 1989. Morphometrical and cell kinetic studies of normal human colorectal mucosa. Comparison between the proximal and the distal large intestine. Acta Pathol Jpn 39:725–730.

Babel I, Barderas R, Díaz-Uriarte R, Martínez-Torrecuadrada JL, Sánchez-Carbayo M, Casal JI. 2009. Identification of tumor-associated autoantigens for the diagnosis of colorectal cancer in serum using high density protein microarrays. Mol Cell Proteomics 8:2382–2395.

Baldus SE, Schaefer KL, Engers R, Hartleb D, Stoecklein NH, Gabbert HE. 2010. Prevalence and heterogeneity of KRAS, BRAF, and PIK3CA mutations in primary colorectal adenocarcinomas and their corresponding metastases. Clin Cancer Res 16:790–799.

Boland CR, Sato J, Appelman HD, Bresalier RS, Feinberg AP. 1995. Microallelotyping defines the sequence and tempo of allelic losses at tumour suppressor gene loci during colorectal cancer progression. Nat Med 1:902–909.

Cardoso J, Molenaar L, de Menezes RX, Rosenberg C, Morreau H, Möslein G, Fodde R, Boer JM. 2004. Genomic profiling by DNA amplification of laser capture microdissected tissues and array CGH. Nucleic Acids Res 32:e146

Chen YG, Wang Q, Lin SL, Chang CD, Chuang J, Ying SY. 2006. Activin signaling and its role in regulation of cell proliferation, apoptosis, and carcinogenesis. Exp Biol Med 231:534–544.

Cheng YW, Pincas H, Bacolod MD, Schemmann G, Giardina SF, Huang J, Barral S, Idrees K, Khan SA, Zeng Z, Rosenberg S, Notterman DA, Ott J, Paty P, Barany F. 2008. CpG island methylator phenotype associates with low-degree chromosomal abnormalities in colorectal cancer. Clin Cancer Res 14: 6005–6013.

Diep CB, Kleivi K, Ribeiro FR, Teixeira MR, Lindgj?rde OC, Lothe RA. 2006. The order of genetic events associated with colorectal cancer progression inferred from meta-analysis of copy number changes. Genes Chromosomes Cancer 45:31–41.

Dix B, Robbins P, Carrello S, House A, Iacopetta B. 1994. Comparison of p53 gene mutation and protein overexpression in colorectal carcinomas. Br J Cancer 70:585–590.

Douglas EJ, Fiegler H, Rowan A, Halford S, Bicknell DC, Bodmer W, Tomlinson IP, Carter NP. 2004. Array comparative genomic hybridization analysis of colorectal cancer cell lines and primary carcinomas. Cancer Res 64:4817–4825.

Fearon ER, Vogelstein B. 1990. A genetic model for colorectal tumorigenesis. Cell 61:759–767.

Gaasenbeek M, Howarth K, Rowan AJ, Gorman PA, Jones A, Chaplin T, Liu Y, Bicknell D, Davison EJ, Fiegler H, Carter NP, Roylance RR, Tomlinson IP. 2006. Combined array-comparative genomic hybridization and single-nucleotide polymorphism-loss of heterozygosity analysis reveals complex changes and multiple forms of chromosomal instability in colorectal cancers. Cancer Res 66:3471–3479.

Goel A, Nagasaka T, Arnold CN, Inoue T, Hamilton C, Niedzwiecki D, Compton C, Mayer RJ, Goldberg R, Bertagnolli MM, Boland CR. 2007. The CpG island methylator phenotype and chromosomal instability are inversely correlated in sporadic colorectal cancer. Gastroenterology 132:127–138.

Gray JW. 2003. Evidence emerges for early metastasis and parallel evolution of primary and metastatic tumors. Cancer Cell 4:4–6.

Grady WM, Carethers J. 2008. Genomic and epigenetic instability in colorectal cancer pathogenesis. Gastroenterology 135: 1079–1099.

Habano W, Sugai T, Nakamura S, Yoshida T. 1996. A novel method for gene analysis of colorectal carcinomas using a crypt isolation technique. Lab Invest 74:933–940.

Hermsen M, Postma C, Baak J, Weiss M, Rapallo A, Sciutto A, Roemen G, Arends JW, Williams R, Giaretti W, De Goeij A, Meijer G. 2002. Colorectal adenoma to carcinoma progression follows multiple pathways of chromosomal instability. Gastroenterology 123:1109–1119.

Hinoue T, Weisenberger DJ, Lange CP, Shen H, Byun HM, Van Den Berg D, Malik S, Pan F, Noushmehr H, van Dijk CM, Tollenaar RA, Laird PW. 2012. Genome-scale analysis of aberrant DNA methylation in colorectal cancer. Genome Res 22:271–282.

Igarashi S, Suzuki H, Niinuma T, Shimizu H, Nojima M, Iwaki H, Nobuoka T, Nishida T, Miyazaki Y, Takamaru H, Yamamoto E, Yamamoto H, Tokino T, Hasegawa T, Hirata K, Imai K, Toyota M, Shinomura Y. 2010. A novel correlation between LINE-1 hypomethylation and the malignancy of gastrointestinal stromal tumors. Clin Cancer Res 16:5114–5123.

Issa JP. 2008. Colon cancer: It's CIN or CIMP. Clin Cancer Res 14:5939–5940.

Japanese Society for Cancer of the Colon and Rectum. 1997. Japanese Classification of Colorectal Carcinoma. Tokyo: Kanehara, pp.30–63.

Jass JR. 2007. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology 50:113–130.

Jhawer M, Goel S, Wilson AJ, Montagna C, Ling YH, Byun DS, Nasser S, Arango D, Shin J, Klampfer L, Augenlicht LH, Perez-Soler R, Mariadason JM. 2008. PIK3CA mutation/PTEN expression status predicts response of colon cancer cells to the epidermal growth factor receptor inhibitor cetuximab. Cancer Res 68:1953–1961.

Jones AM, Douglas EJ, Halford SE, Fiegler H, Gorman PA, Roylance RR, Carter NP, Tomlinson IP. 2005. Array-CGH analysis of microsatellite-stable, near-diploid bowel cancers and comparison with other types of colorectal carcinoma. Oncogene 24:118–129.

Jover R, Nguyen TP, Pérez-Carbonell L, Zapater P, Payá A, Alenda C, Rojas E, Cubiella J, Balaguer F, Morillas JD, Clofent J, Bujanda L, Reñé JM, Bessa X, Xicola RM, Nicolás-Pérez D, Castells A, Andreu M, Llor X, Boland CR, Goel A. 2011. 5-Fluorouracil adjuvant chemotherapy does not increase survival in patients with CpG island methylator phenotype colorectal cancer. Gastroenterology 140:1174–1181.

Ju HX, An B, Okamoto Y, Shinjo K, Kanemitsu Y, Komori K, Hirai T, Shimizu Y, Sano T, Sawaki A, Tajika M, Yamao K, Fujii M, Murakami H, Osada H, Ito H, Takeuchi I, Sekido Y, Kondo Y. 2011. Distinct profiles of epigenetic evolution between colorectal cancers with and without metastasis. Am J Pathol 178:1835–1846.

Jung B, Doctolero RT, Tajima A, Nguyen AK, Keku T, Sandler RS, Carethers JM. 2004. Loss of activin receptor type 2 protein expression in microsatellite unstable colon cancers. Gastroenterology 126:654–659.

Jung BH, Beck SE, Cabral J, Chau E, Cabrera BL, Fiorino A, Smith EJ, Bocanegra M, Carethers JM. 2007. Activin type 2 receptor restoration in MSI-H colon cancer suppresses growth and enhances migration with activin. Gastroenterology 132: 633–644.

Meijer GA, Hermsen MA, Baak JP, van Diest PJ, Meuwissen SG, Beliën JA, Hoovers JM, Joenje H, Snijders PJ, Walboomers JM. 1998. Progression from colorectal adenoma to carcinoma is associated with non-random chromosomal gains as detected by comparative genomic hybridisation. J Clin Pathol 51:901–909.

Miyazaki M, Furuya T, Shiraki A, Sato T, Oga A, Sasaki K. 1999. The relationship of DNA ploidy to chromosomal instability in primary human colorectal cancers. Cancer Res 59: 5283–5285.

Nakamura S, Goto J, Kitayama M, Kino I. 1994. Application of the crypt-isolation technique to flow-cytometric analysis of DNA content in colorectal neoplasms. Gastroenterology 106:100–107.

Nakao K, Mehta KR, Fridlyand J, Moore DH, Jain AN, Lafuente A, Wiencke JW, Terdiman JP, Waldman FM. 2004. High-resolution analysis of DNA copy number alterations in colorectal cancer by array-based comparative genomic hybridization. Carcinogenesis 25:1345–1357.

Ogino S, Kawasaki T, Kirkner GJ, Loda M, Fuchs CS. 2006. CpG island methylator phenotype-low (CIMP-low) in colorectal cancer: possible associations with male sex and KRAS mutations. J Mol Diagn 8:582–588.

Postma C, Koopman M, Buffart TE, Eijk PP, Carvalho B, Peters GJ, Ylstra B, van Krieken JH, Punt CJ, Meijer GA. 2009. DNA copy number profiles of primary tumors as predictors of response to chemotherapy in advanced colorectal cancer. Ann Oncol 20:1048–1056.

Poulogiannis G, Ichimura K, Hamoudi RA, Luo F, Leung SY, Yuen ST, Harrison DJ, Wyllie AH, Arends MJ. 2010. Prognostic relevance of DNA copy number changes in colorectal cancer. J Pathol 220:338–347.

Pritchard CC, Grady WM. 2011. Colorectal cancer molecular biology moves into clinical practice. Gut 60:116–129.

Rajagopalan H, Lengauer C. 2004. Aneuploidy and cancer. Nature 432:338–341.

Ried T, Knutzen R, Steinbeck R, Blegen H, Schröck E, Heselmeyer K, du Manoir S, Auer G. 1996. Comparative genomic hybridization reveals a specific pattern of chromosomal gains and losses during the genesis of colorectal tumors. Genes Chromosomes Cancer 15:234–245.

Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, Yan H, Gazdar A, Powell SM, Riggins GJ, Willson JK, Markowitz S, Kinzler KW, Vogelstein B, Velculescu VE. 2004. High frequency of mutations of the PIK3CA gene in human cancers. Science 304:554.

Sheffer M, Bacolod MD, Zuk O, Giardina SF, Pincas H, Barany F, Paty PB, Gerald WL, Notterman DA, Domany E.Association of survival and disease progression with chromosomal instability: a genomic exploration of colorectal cancer.2009.Proc Natl Acad Sci USA 106:7131–7136.

Shen L, Catalano PJ, Benson AB III, O'Dwyer P, Hamilton SR, Issa JP. 2007a. Association between DNA methylation and shortened survival in patients with advanced colorectal cancer treated with 5-fluorouracil based chemotherapy. Clin Cancer Res 13:6093–6098.

Shen L, Toyota M, Kondo Y, Lin E, Zhang L, Guo Y, Hernandez NS, Chen X, Ahmed S, Konishi K, Hamilton SR, Issa JP. 2007b. Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. Proc Natl Acad Sci USA 104:18654–18659.

Smith G, Carey FA, Beattie J, Wilkie MJ, Lightfoot TJ, Coxhead J, Garner RC, Steele RJ, Wolf CR. 2002. Mutations in APC, Kirsten-ras, and p53 - alternative genetic pathways to colorectal cancer. Proc Natl Acad Sci USA 99:9433–9438.

Sugai T, Habano W, Nakamura S, Uesugi N, Sasou S, Itoh C. 2000. A unique method for mutation analysis of tumor suppressor genes in colorectal carcinomas using a crypt isolation technique. Arch Pathol Lab Med 124:382–386.

Sugai T, Habano W, Jiao YF, Suzuki M, Takagi R, Otsuka K, Higuchi T, Nakamura S. 2005. Analysis of allelic imbalances at multiple cancer-related chromosomal loci and microsatellite instability within the same tumor using a single tumor gland from colorectal carcinomas. Int J Cancer 114:337–345.

Threadgill DW. 2005. Metastatic potential as a heritable trait. Nat Genet 37:1026–1027.

Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. 1999. CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci USA 96:8681–8686.

Toyota M, Suzuki H, Sasaki Y, Maruyama R, Imai K, Shinomura Y, Tokino T. 2008. Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer. Cancer Res 68:4123–4132.

Turnbull RB Jr, Kyle K, Watson FR, Spratt J. 1967. Cancer of the colon: the influence of the no-touch isolation technic on survival rates. Ann Surg 166:420–427.

Walther A, Houlston R, Tomlinson I. 2008. Association between chromosomal instability and prognosis in colorectal cancer: a meta-analysis. Gut 57:941–950.

Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH, Widschwendter M, Weener D, Buchanan D, Koh H, Simms L, Barker M, Leggett B, Levine J, Kim M, French AJ, Thibodeau SN, Jass J, Haile R, Laird PW. 2006. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet 38:787–793.

Yonesaka K, Zejnullahu K, Okamoto I, Satoh T, Cappuzzo F, Souglakos J, Ercan D, Rogers A, Roncalli M, Takeda M, Fujisaka Y, Philips J, Shimizu T, Maenishi O, Cho Y, Sun J, Destro A, Taira K, Takeda K, Okabe T, Swanson J, Itoh H, Takada M, Lifshits E, Okuno K, Engelman JA, Shivdasani RA, Nishio K, Fukuoka M, Varella-Garcia M, Nakagawa K, Jänne PA. 2011. Activation of ERBB2 signaling causes resistance to the EGFR-directed therapeutic antibody cetuximab. Sci Transl Med 3:99ra86.

201313002B（3/3）

厚生労働科学研究費補助金

第３次対がん総合戦略研究事業

ヒトがんにおけるエピジェネティックな異常の解明と応用に関する研究

平成２２年度～２５年度　総合研究報告書

研究代表者　牛島　俊和

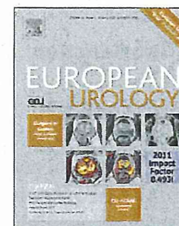
平成２６年（２０１４）年　5月

３／３冊

Author's personal copy

EUROPEAN UROLOGY 63 (2013) 1091–1100

available at www.sciencedirect.com
journal homepage: www.europeanurology.com



Urothelial Cancer

Methylation of a Panel of MicroRNA Genes Is a Novel Biomarker for Detection of Bladder Cancer

Takashi Shimizu[a,b], *Hiromu Suzuki*[b,c,*], *Masanori Nojima*[c,d], *Hiroshi Kitamura*[a], *Eiichiro Yamamoto*[b,c], *Reo Maruyama*[b,c], *Masami Ashida*[b], *Tomo Hatahira*[b], *Masahiro Kai*[b], *Naoya Masumori*[a], *Takashi Tokino*[e], *Kohzoh Imai*[f], *Taiji Tsukamoto*[a,**], *Minoru Toyota*[b]

[a] Department of Urology, Sapporo Medical University, Sapporo, Japan; [b] Department of Molecular Biology, Sapporo Medical University, Sapporo, Japan; [c] First Department of Internal Medicine, Sapporo Medical University, Sapporo, Japan; [d] Department of Public Health, Sapporo Medical University, Sapporo, Japan; [e] Medical Genome Science, Research Institute for Frontier Medicine, Sapporo Medical University, Sapporo, Japan; [f] Division of Novel Therapy for Cancer, The Advanced Clinical Research Center, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

Article info

Article history:
Accepted November 13, 2012
Published online ahead of print on November 23, 2012

Keywords:
Biomarker
Bladder cancer
DNA methylation
MicroRNA
Urinary test

Abstract

Background: Dysregulation of microRNAs (miRNAs) has been implicated in bladder cancer (BCa), although the mechanism is not fully understood.
Objective: We aimed to explore the involvement of epigenetic alteration of miRNA expression in BCa.
Design, setting, and participants: Two BCa cell lines (T24 and UM-UC-3) were treated with 5-aza-2′-deoxycytidine (5-aza-dC) and 4-phenylbutyric acid (PBA), after which their miRNA expression profiles were analyzed using a TaqMan array (Life Technologies, Carlsbad, CA, USA). Bisulfite pyrosequencing was used to assess miRNA gene methylation in 5 cancer cell lines, 83 primary tumors, and 120 preoperative and 47 postoperative urine samples.
Outcome measurements and statistical analysis: Receiver operating characteristic (ROC) curve analysis was used to assess the diagnostic performance of the miRNA gene panel.
Results and limitations: Of 664 miRNAs examined, 146 were upregulated by 5-aza-dC plus PBA. CpG islands were identified in the proximal upstream of 23 miRNA genes, and 12 of those were hypermethylated in cell lines. Among them, miR-137, miR-124-2, miR-124-3, and miR-9-3 were frequently and tumor-specifically methylated in primary cancers (miR-137: 68.7%; miR-124-2: 50.6%; miR-124-3: 65.1%; miR-9-3: 45.8%). Methylation of the same four miRNAs in urine specimens enabled BCa detection with 81% sensitivity and 89% specificity; the area under the ROC curve was 0.916. Ectopic expression of silenced miRNAs in BCa cells suppressed growth and cell invasion.
Conclusions: Our results indicate that epigenetic silencing of miRNA genes may be involved in the development of BCa and that methylation of miRNA genes could be a useful biomarker for cancer detection.
© 2012 European Association of Urology. Published by Elsevier B.V. All rights reserved.

* Corresponding author. Department of Molecular Biology, Sapporo Medical University, S1, W17, Chuo-Ku, Sapporo 060-8556, Japan. Tel. +81 11 611 2111; Fax: +81 11 622 1918.
** Corresponding author. Department of Urology, Sapporo Medical University, S1, W16, Chuo-Ku, Sapporo 060-8543, Japan. Tel. +81 11 611 2111; Fax: +81 11 612 2709.
E-mail addresses: hsuzuki@sapmed.ac.jp (H. Suzuki), taijit@sapmed.ac.jp (T. Tsukamoto).

0302-2838/$ – see back matter © 2012 European Association of Urology. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.eururo.2012.11.030