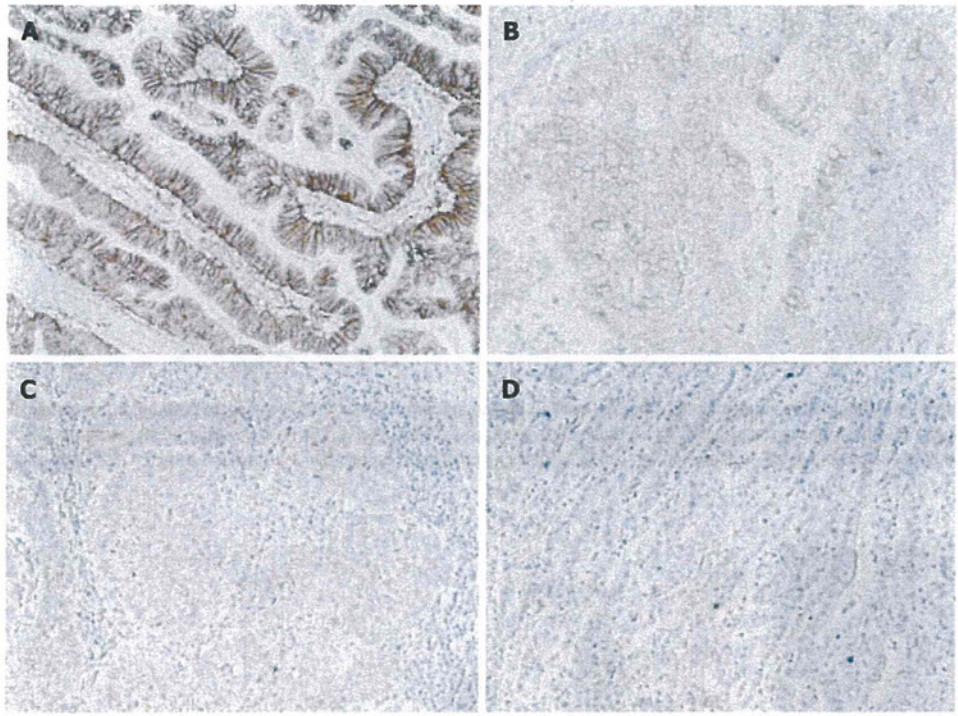

Figure 1 Immunohistochemical analysis of human epidermal growth factor receptor 2 in gastric cancer tissues. A: Human epidermal growth factor receptor 2 (HER2) 3+; B: HER2 2+; C: HER2 1+; D: HER2 0. Original magnification, ×200.

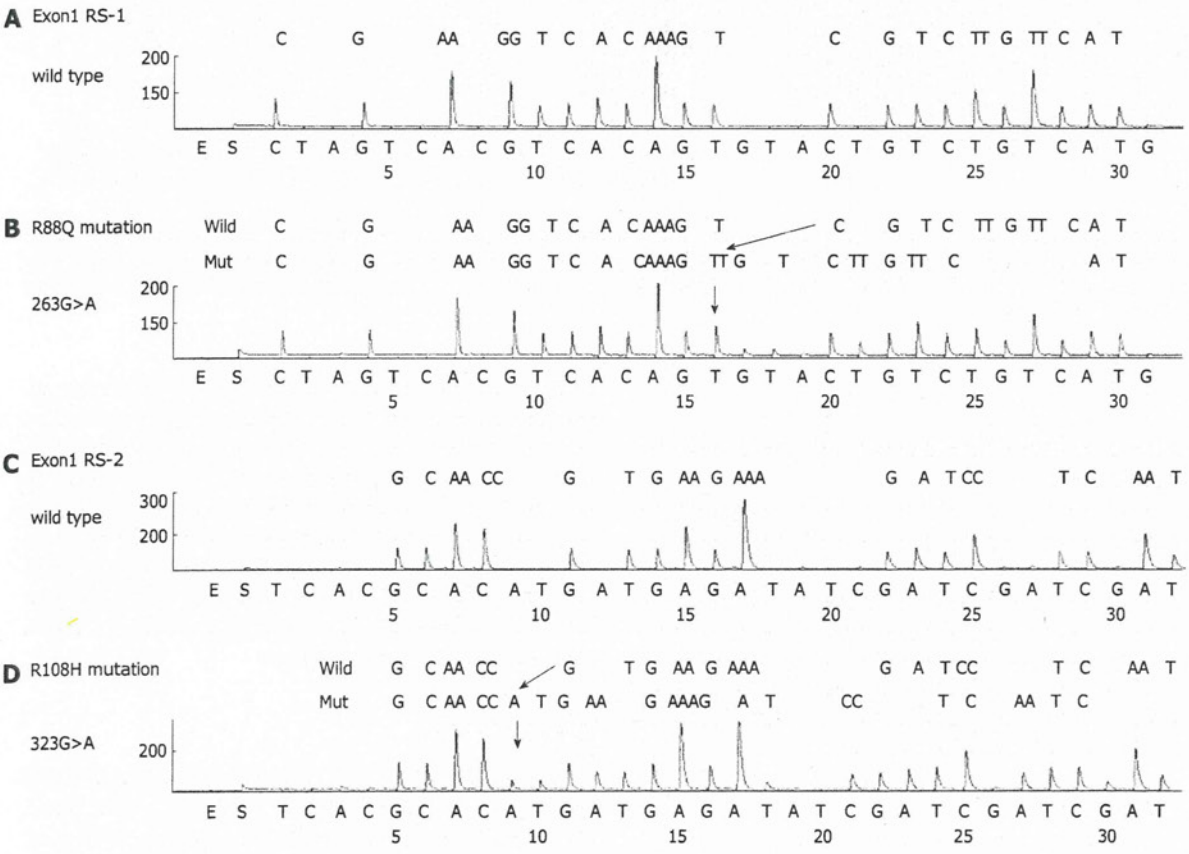

Figure 2 Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide mutations detected by pyrosequencing in gastric cancer tissues. A: Exon1 RS1 wild type; B: 263G>A (R88Q) mutation; C: Exon1 RS2 wild type; D: 323G>A (R108H) mutation.

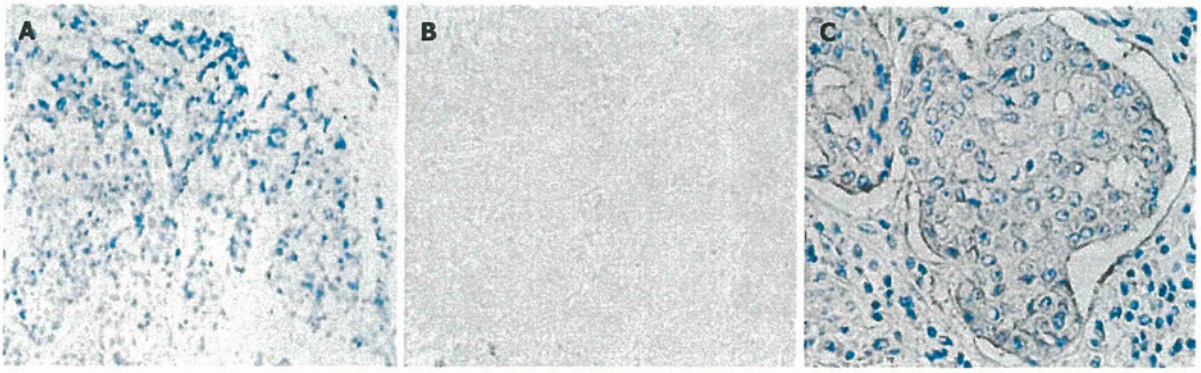

Figure 3 *In situ* hybridization analysis of Epstein-Barr virus-encoded small RNA-1 and human epidermal growth factor receptor 2 immunohistochemical expression in gastric cancer tissues. A: Gastric adenocarcinoma positive for Epstein-Barr virus-encoded small RNA-1 (EBER-1); B: Gastric adenocarcinoma negative for EBER-1; C: Immunohistochemical analysis of human epidermal growth factor receptor 2 (HER2) in an Epstein-Barr virus-positive and HER2-positive case. Original magnification, ×200.

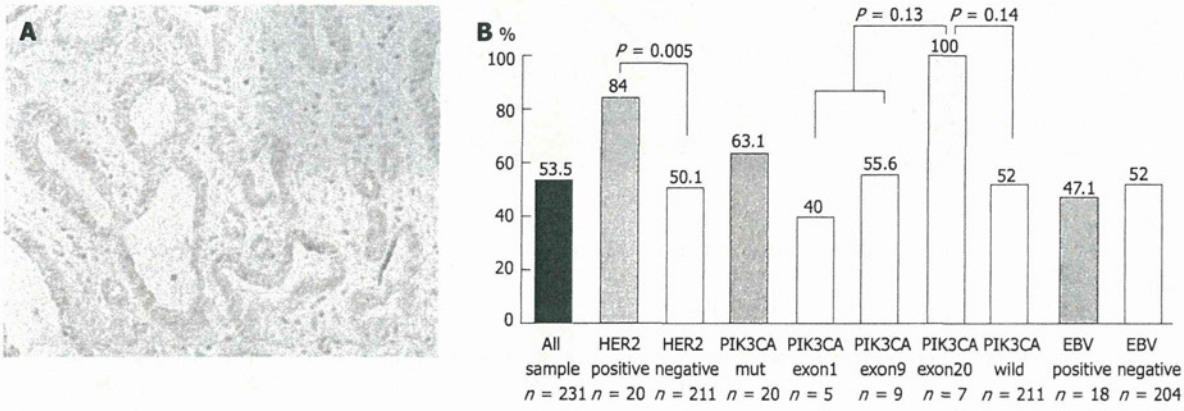

Figure 4 Immunohistochemical analysis and assessment of phospho Akt positivity based on molecular alterations in gastric cancer tissues. A: Gastric adenocarcinoma showing phospho Akt (pAkt) positivity. Original magnification, ×200; B: pAkt expression significantly correlates with human epidermal growth factor receptor 2 (HER2) overexpression ($P < 0.01$) but not with phosphatidylinositol 3-kinase, catalytic, alpha polypeptide (PIK3CA) mutations ($P = 0.37$) or Epstein-Barr virus (EBV) infection ($P = 0.69$).

0.36-2.31, $P = 0.98$).

Association of HER2 overexpression, PIK3CA mutations and EBV infection

One of our cases showed both HER2 overexpression and EBV infection and 3 cases showed both PIK3CA mutations and EBV infection. However there were no cases showing both PIK3CA mutations and HER2 overexpression. Three of the 4 cases were positive also for pAkt expression. PIK3CA mutations were present in 3 EBV-positive cancers, including 2 cases of LELC (2/5, 40%). One EBV-positive cancer with a PIK3CA mutation (H1047R) was MSI-positive.

pAkt expression

pAkt expression was positive in 119 (53%) of our cases but this showed no correlation with clinicopathological characteristics (Figure 4A). On the other hand, pAkt expression was found to be significantly correlated with HER2 overexpression (16/19 *vs* 103/204, $P < 0.01$) but not with PIK3CA mutations (12/19 *vs* 107/204, $P = 0.37$) or EBV infection (8/17 *vs* 103/198, $P = 0.69$) (Table 2). The frequency of pAkt expression was higher in cancers with exon20 mutations (100%) than in those with exon1 (40%) or exon9 (56%) mutations of PIK3CA, although this difference did not reach statistical significance (Figure 4B). The five-year survival rates were 37% in patients with pAkt expression and 59% in those without pAkt expression (HR 1.75; 95%CI: 1.12-2.80, $P = 0.02$) (Figure 5). Hence, pAkt expression significantly correlates with a poor prognosis in gastric cancer.

DISCUSSION

In our present study, we systematically characterized HER2 expression, PIK3CA mutations and EBV infection, all of which are involved in the PI3K-Akt signaling pathway, in a large cohort of patients with gastric cancer ($n = 231$). We aimed to determine the prevalence of these characteristics with a high level of precision and to correlate them with clinicopathological and molecular features, such as MSI and pAkt expression.

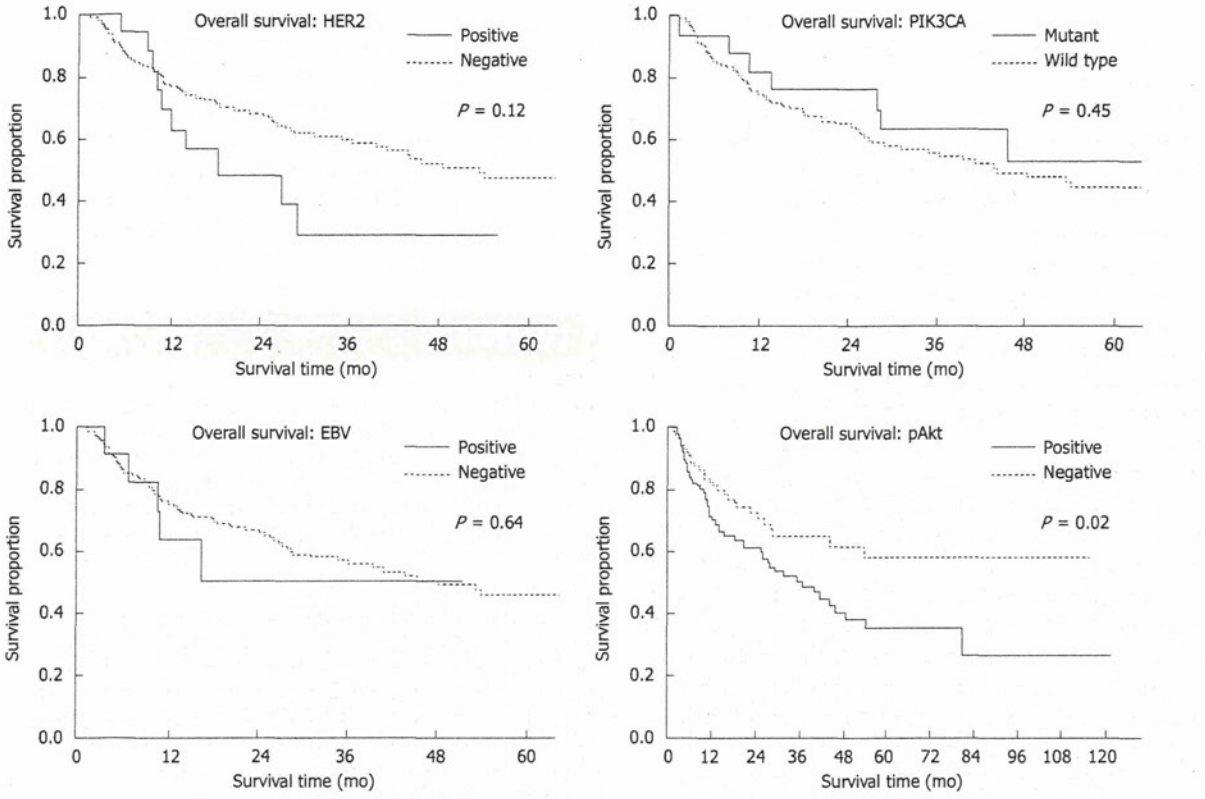

Figure 5 Survival analysis of gastric cancer patients. Three year survival of human epidermal growth factor receptor 2 (HER2)-positive *vs* HER2-negative, 29.1 mo *vs* 59.4 mo; Phosphatidylinositol 3-kinase, catalytic, alpha polypeptide (PIK3CA) mutation *vs* wild type, 63.7 mo *vs* 56.3 mo; Epstein-Barr virus (EBV)-positive *vs* EBV-negative, 51.3 mo *vs* 57.6 mo; And phospho Akt (pAkt)-positive *vs* pAkt-negative, 50.7 mo *vs* 64.8 mo. Five year survival of pAkt-positive *vs* pAkt-negative cases, 35.5 mo *vs* 58.1 mo.

HER2 overexpression (IHC 3+) was present in 20 samples (8.4%), a value that is within the range (7%-34%) reported in the current literature[5-9]. HER2 overexpression was found to significantly correlate with the intestinal histological type. Hence, the frequency of HER2 expression may depend on, at least in part, the distribution of histology in a cohort of gastric cancer samples. Some studies have suggested that HER2 positivity in gastric cancer is associated with poor outcomes and aggressive disease, but the results are conflicting. We found for the first time in our present analyses that HER2 overexpression significantly correlates with pAkt expression in gastric cancer tissues. Moreover, pAkt expression correlated with a poor prognosis in these patients. Thus, the HER2-Akt axis may play an important role in gastric cancer.

Pyrosequencing-based methods facilitate the identification of low-frequency tumor mutations and allow a more accurate assessment of tumor mutation burden[17,23,24]. We characterized PIK3CA mutations in gastric cancer tissues using pyrosequencing for the first time. The overall prevalence of PIK3CA mutations was found in our analysis to be 8.7%, a value that is within the previously reported range (4% to 25%)[10,12-15]. The mutation frequency was found to be high (21.4%) in T4 cancers and low (6.4%) in T2 cancers in our sample cohort. Thus, PIK3CA mutations appear to be late events in gastric carcinogenesis, leading to tumor progression. These patients might therefore be appropriate for targeted therapies directed against the PI3K pathway.

The most common PIK3CA mutation found in our analysis was H1047R, which was also found previously[15]. Importantly, two new types of mutations were found in exon1. To our knowledge, PIK3CA mutations involving residues 88 and 108 (R88Q and R108H) have been never reported previously in gastric cancer, nor described in the COSMIC database, despite the large number of previous studies in which this region was investigated. These mutations have been detected in several other types of cancer tissues[25]. Importantly also, these mutations have been reported to be gain-of-function[26-28]. Our present results thus have potential clinical implications since the mutational status of PIK3CA could stratify patients for genotype-based molecular therapies targeting the PI3K pathway. Hence, exon1 of PIK3CA should be analyzed in gastric cancer patients in these clinical settings.

PIK3CA mutations were found to be significantly associated with the MSI phenotype in our experiments. An association between PIK3CA mutations and MSI has been reported, or at least suggested, for both gastric and colon cancers[12,13,29]. We found in our present study that PIK3CA mutations in cancers with MSI are distributed in exon1, exon9 and exon20. These results further sup-

port the notion that PIK3CA is one of the most important oncogenes activated by missense mutations in MSI-positive gastric cancers.

The frequency of pAkt expression was found to be higher in cancers with exon20 mutations (100%) than in those with exon1 (40%) or exon9 (56%) mutations in PIK3CA. These results further support the notion that the functional significance of PIK3CA mutations depends on the mutation type and that the H1047R hotspot mutation has high oncogenic activity.

The previous ToGA study has shown that the addition of trastuzumab to the chemotherapeutic regimen improves survival in patients with advanced gastric or gastroesophageal junction cancer[5,6]. PIK3CA mutation is one of the mechanisms underlying the resistance to trastuzumab in breast cancer[30]. Trastuzumab is likely to be effective for HER2-overexpressing breast cancers with no PIK3CA mutations, with possible rescue using HER2-TKIs in cases of relapse[31]. For HER2-overexpressing breast cancer with PIK3CA mutations, inhibitors against molecules of the PI3K pathway are possibly more effective than anti-HER2 agents, which are unlikely to be beneficial[32]. In our present study, PIK3CA mutations were not found in gastric cancers with HER2 overexpression. Thus, it is unlikely that PIK3CA mutation is a major mechanism underlying the resistance to trastuzumab in gastric cancer.

HER2 overexpression was found in only one of the 18 EBV-positive gastric cancers in our sample cohort. This result can be explained, at least in part, by the fact that HER2 overexpression and EBV infection significantly correlate with intestinal and diffuse histological types, respectively. On the other hand, PIK3CA mutations were identified in 3 EBV-positive cancers, including 2 cases of LELC (2/5, 40%). Although not analyzed in our current study, EBV infection reportedly inactivates PTEN through the CpG island methylation of its promoter in EBV-associated gastric cancer[21]. Thus, alterations in the PI3K-Akt signaling pathway in EBV-positive gastric cancers may differ from those in EBV-negative cancers.

Finally, pAkt expression was found to correlate with a poor prognosis in gastric cancer. A significant association between increased pAkt expression and poor prognosis has been reported previously in patients with T3/T4 gastric cancer but not in those with T1/T2 cancer[33]. It has been reported also that pAkt expression is associated with increased resistance to multiple chemotherapeutic agents in gastric cancer patients, when chemotherapeutic sensitivities were tested using MTT assays[34]. Thus, Akt activation appears to lead to a poor prognosis and resistance to chemotherapeutic agents in gastric cancer. A positive correlation between a decrease in the pAkt levels after gefitinib administration and tumor apoptotic index in gastric cancer has also been reported[35]. Further analyses regarding the pAkt status in cancer tissues before and after chemotherapy and molecular targeted therapy will be necessary. Not all Akt activation events can be explained by HER2 expression, PIK3CA mutations, and EBV infection in gastric cancer. We have reported previously that a dominant negative insulin-like growth factor (IGF)-1 receptor blocks the Akt-1 activation induced by IGF-1 and IGF-2 in gastric cancer cell lines[36]. Thus, molecular alterations, such as the overexpression of IGF-1 receptor, might be involved in the activation of Akt in gastric cancer and this issue needs to be clarified in the near future.

COMMENTS

Background

Personalized therapy has begun also in advanced gastric cancer through the use of trastuzumab, an anti-human epidermal growth factor receptor 2 (HER2) antibody. Many drugs targeting the phosphatidylinositol 3-kinase (PI3K)-Akt pathway have now been developed and clinical trials are ongoing. An appropriate biomarker is necessary for successful molecular targeted therapy. The alterations of molecules in the PI3K-Akt pathway could be a good biomarker for such drugs.

Research frontiers

Various alterations, such as activation of growth factor receptors, PI3K, catalytic, alpha polypeptide (PIK3CA) mutations and Epstein-Barr virus (EBV) infection lead to activation of the PI3K-Akt signaling pathway. However, clinicopathological and molecular correlates among such alterations have not been clearly addressed. In the present study, the authors identify new clinicopathological and molecular correlations between HER2 expression, PIK3CA mutations, EBV infection and phospho Akt (pAkt) expression in gastric cancer.

Innovations and breakthroughs

This is the first study to systematically characterize HER2 expression, PIK3CA mutations and EBV infection, all of which are involved in the PI3K-Akt signaling pathway, in a large cohort of patients with gastric cancer. The prevalence of these characteristics was thereby determined with a high level of precision and correlations with the clinicopathological and molecular features of gastric cancers, such as microsatellite instability and pAkt expression, could be assessed accurately for the first time.

Applications

The results have potentially important clinical implications since the mutational status of PIK3CA can be used to stratify cancer patients for genotype-based molecular therapies that target the HERs-PI3K pathway.

Terminology

PI3K-Akt pathway: Akt is believed to transduce the major downstream PI3K signals in cancer. Akt regulates cell growth and survival pathways by phosphorylating substrates such as GSK3, forkhead transcription factors, and the TSC2 tumor suppressor protein; PIK3CA: PIK3CA encodes a key enzymatic subunit of PI3K. Gain of function mutations in PIK3CA occur frequently in several cancer types. Hotspots of PIK3CA mutations are located in exons 9 and 20.

Peer review

The authors investigated HER2 expression, PIK3CA mutations and EBV infection in patients with gastric cancer. The results demonstrated that pAkt expression significantly correlates with the prognosis and the HER2 expression status in gastric cancer. This article is important for the further development of molecular targeted therapy in patients with advanced gastric cancer.

REFERENCES

1 **Hamilton JP**, Sato F, Greenwald BD, Suntharalingam M, Krasna MJ, Edelman MJ, Doyle A, Berki AT, Abraham JM, Mori Y, Kan T, Mantzur C, Paun B, Wang S, Ito T, Jin Z, Meltzer SJ. Promoter methylation and response to chemotherapy and radiation in esophageal cancer. *Clin Gastroenterol Hepatol* 2006; **4**: 701-708

2 **Tamura G**. Alterations of tumor suppressor and tumor-related genes in the development and progression of gastric

cancer. *World J Gastroenterol* 2006; **12**: 192-198
3 **Engelman JA**. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. *Nat Rev Cancer* 2009; **9**: 550-562
4 **Mueller A**, Bachmann E, Linnig M, Khillimberger K, Schimanski CC, Galle PR, Moehler M. Selective PI3K inhibition by BKM120 and BEZ235 alone or in combination with chemotherapy in wild-type and mutated human gastrointestinal cancer cell lines. *Cancer Chemother Pharmacol* 2012; **69**: 1601-1615
5 **Bang YJ**, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, Lehle M, Rüschoff J, Kang YK. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet* 2010; **376**: 687-697
6 **Sawaki A**, Ohashi Y, Omuro Y, Satoh T, Hamamoto Y, Boku N, Miyata Y, Takiuchi H, Yamaguchi K, Sasaki Y, Nishina T, Satoh A, Baba E, Tamura T, Abe T, Hatake K, Ohtsu A. Efficacy of trastuzumab in Japanese patients with HER2-positive advanced gastric or gastroesophageal junction cancer: a subgroup analysis of the Trastuzumab for Gastric Cancer (ToGA) study. *Gastric Cancer* 2012; **15**: 313-322
7 **Gravalos C**, Jimeno A. HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target. *Ann Oncol* 2008; **19**: 1523-1529
8 **Hofmann M**, Stoss O, Shi D, Büttner R, van de Vijver M, Kim W, Ochiai A, Rüschoff J, Henkel T. Assessment of a HER2 scoring system for gastric cancer: results from a validation study. *Histopathology* 2008; **52**: 797-805
9 **Tanner M**, Hollmén M, Junttila TT, Kapanen AI, Tommola S, Soini Y, Helin H, Salo J, Joensuu H, Sihvo E, Elenius K, Isola J. Amplification of HER-2 in gastric carcinoma: association with Topoisomerase IIalpha gene amplification, intestinal type, poor prognosis and sensitivity to trastuzumab. *Ann Oncol* 2005; **16**: 273-278
10 **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. High frequency of mutations of the PIK3CA gene in human cancers. *Science* 2004; **304**: 554
11 **Lee JW**, Soung YH, Kim SY, Lee HW, Park WS, Nam SW, Kim SH, Lee JY, Yoo NJ, Lee SH. PIK3CA gene is frequently mutated in breast carcinomas and hepatocellular carcinomas. *Oncogene* 2005; **24**: 1477-1480
12 **Li VS**, Wong CW, Chan TL, Chan AS, Zhao W, Chu KM, So S, Chen X, Yuen ST, Leung SY. Mutations of PIK3CA in gastric adenocarcinoma. *BMC Cancer* 2005; **5**: 29
13 **Velho S**, Oliveira C, Ferreira A, Ferreira AC, Suriano G, Schwartz S, Duval A, Carneiro F, Machado JC, Hamelin R, Seruca R. The prevalence of PIK3CA mutations in gastric and colon cancer. *Eur J Cancer* 2005; **41**: 1649-1654
14 **Ligresti G**, Militello L, Steelman LS, Cavallaro A, Basile F, Nicoletti F, Stivala F, McCubrey JA, Libra M. PIK3CA mutations in human solid tumors: role in sensitivity to various therapeutic approaches. *Cell Cycle* 2009; **8**: 1352-1358
15 **Barbi S**, Cataldo I, De Manzoni G, Bersani S, Lamba S, Mattuzzi S, Bardelli A, Scarpa A. The analysis of PIK3CA mutations in gastric carcinoma and metanalysis of literature suggest that exon-selectivity is a signature of cancer type. *J Exp Clin Cancer Res* 2010; **29**: 32
16 **Kataoka Y**, Mukohara T, Shimada H, Saijo N, Hirai M, Minami H. Association between gain-of-function mutations in PIK3CA and resistance to HER2-targeted agents in HER2-amplified breast cancer cell lines. *Ann Oncol* 2010; **21**: 255-262
17 **Weidlich S**, Walsh K, Crowther D, Burczynski ME, Feuerstein G, Carey FA, Steele RJ, Wolf CR, Miele G, Smith G. Pyrosequencing-based methods reveal marked inter-individual differences in oncogene mutation burden in human colorectal tumours. *Br J Cancer* 2011; **105**: 246-254
18 **Kang GH**, Lee S, Kim WH, Lee HW, Kim JC, Rhyu MG, Ro JY. Epstein-barr virus-positive gastric carcinoma demonstrates frequent aberrant methylation of multiple genes and constitutes CpG island methylator phenotype-positive gastric carcinoma. *Am J Pathol* 2002; **160**: 787-794
19 **Kusano M**, Toyota M, Suzuki H, Akino K, Aoki F, Fujita M, Hosokawa M, Shinomura Y, Imai K, Tokino T. Genetic, epigenetic, and clinicopathologic features of gastric carcinomas with the CpG island methylator phenotype and an association with Epstein-Barr virus. *Cancer* 2006; **106**: 1467-1479
20 **Chang MS**, Uozaki H, Chong JM, Ushiku T, Sakuma K, Ishikawa S, Hino R, Barua RR, Iwasaki Y, Arai K, Fujii H, Nagai H, Fukayama M. CpG island methylation status in gastric carcinoma with and without infection of Epstein-Barr virus. *Clin Cancer Res* 2006; **12**: 2995-3002
21 **Hino R**, Uozaki H, Murakami N, Ushiku T, Shinozaki A, Ishikawa S, Morikawa T, Nakaya T, Sakatani T, Takada K, Fukayama M. Activation of DNA methyltransferase 1 by EBV latent membrane protein 2A leads to promoter hypermethylation of PTEN gene in gastric carcinoma. *Cancer Res* 2009; **69**: 2766-2774
22 **Gori S**, Sidoni A, Colozza M, Ferri I, Mameli MG, Fenocchio D, Stocchi L, Foglietta J, Ludovini V, Minenza E, De Angelis V, Crinò L. EGFR, pMAPK, pAkt and PTEN status by immunohistochemistry: correlation with clinical outcome in HER2-positive metastatic breast cancer patients treated with trastuzumab. *Ann Oncol* 2009; **20**: 648-654
23 **Nosho K**, Kawasaki T, Ohnishi M, Suemoto Y, Kirkner GJ, Zepf D, Yan L, Longtine JA, Fuchs CS, Ogino S. PIK3CA mutation in colorectal cancer: relationship with genetic and epigenetic alterations. *Neoplasia* 2008; **10**: 534-541
24 **Baba Y**, Nosho K, Shima K, Hayashi M, Meyerhardt JA, Chan AT, Giovannucci E, Fuchs CS, Ogino S. Phosphorylated AKT expression is associated with PIK3CA mutation, low stage, and favorable outcome in 717 colorectal cancers. *Cancer* 2011; **117**: 1399-1408
25 **Rudd ML**, Price JC, Fogoros S, Godwin AK, Sgroi DC, Merino MJ, Bell DW. A unique spectrum of somatic PIK3CA (p110alpha) mutations within primary endometrial carcinomas. *Clin Cancer Res* 2011; **17**: 1331-1340
26 **Miyake T**, Yoshino K, Enomoto T, Takata T, Ugaki H, Kim A, Fujiwara K, Miyatake T, Fujita M, Kimura T. PIK3CA gene mutations and amplifications in uterine cancers, identified by methods that avoid confounding by PIK3CA pseudogene sequences. *Cancer Lett* 2008; **261**: 120-126
27 **Oda K**, Okada J, Timmerman L, Rodriguez-Viciana P, Stokoe D, Shoji K, Taketani Y, Kuramoto H, Knight ZA, Shokat KM, McCormick F. PIK3CA cooperates with other phosphatidylinositol 3'-kinase pathway mutations to effect oncogenic transformation. *Cancer Res* 2008; **68**: 8127-8136
28 **Gymnopoulos M**, Elsliger MA, Vogt PK. Rare cancer-specific mutations in PIK3CA show gain of function. *Proc Natl Acad Sci USA* 2007; **104**: 5569-5574
29 **Corso G**, Velho S, Paredes J, Pedrazzani C, Martins D, Milanezi F, Pascale V, Vindigni C, Pinheiro H, Leite M, Marrelli D, Sousa S, Carneiro F, Oliveira C, Roviello F, Seruca R. Oncogenic mutations in gastric cancer with microsatellite instability. *Eur J Cancer* 2011; **47**: 443-451
30 **Berns K**, Horlings HM, Hennessy BT, Madiredjo M, Hijmans EM, Beelen K, Linn SC, Gonzalez-Angulo AM, Stemke-Hale K, Hauptmann M, Beijersbergen RL, Mills GB, van de Vijver MJ, Bernards R. A functional genetic approach identifies the PI3K pathway as a major determinant of trastuzumab resistance in breast cancer. *Cancer Cell* 2007; **12**: 395-402
31 **Wang L**, Zhang Q, Zhang J, Sun S, Guo H, Jia Z, Wang B, Shao Z, Wang Z, Hu X. PI3K pathway activation results in low efficacy of both trastuzumab and lapatinib. *BMC Cancer*

2011; **11**: 248
32 **Crawford A**, Nahta R. Targeting Bcl-2 in Herceptin-Resistant Breast Cancer Cell Lines. *Curr Pharmacogenomics Person Med* 2011; **9**: 184-190
33 **Murakami D**, Tsujitani S, Osaki T, Saito H, Katano K, Tatebe S, Ikeguchi M. Expression of phosphorylated Akt (pAkt) in gastric carcinoma predicts prognosis and efficacy of chemotherapy. *Gastric Cancer* 2007; **10**: 45-51
34 **Oki E**, Baba H, Tokunaga E, Nakamura T, Ueda N, Futatsugi M, Mashino K, Yamamoto M, Ikebe M, Kakeji Y, Maehara Y. Akt phosphorylation associates with LOH of PTEN and leads to chemoresistance for gastric cancer. *Int J Cancer* 2005; **117**: 376-380
35 **Rojo F**, Tabernero J, Albanell J, Van Cutsem E, Ohtsu A, Doi T, Koizumi W, Shirao K, Takiuchi H, Ramon y Cajal S, Baselga J. Pharmacodynamic studies of gefitinib in tumor biopsy specimens from patients with advanced gastric carcinoma. *J Clin Oncol* 2006; **24**: 4309-4316
36 **Min Y**, Adachi Y, Yamamoto H, Imsumran A, Arimura Y, Endo T, Hinoda Y, Lee CT, Nadaf S, Carbone DP, Imai K. Insulin-like growth factor I receptor blockade enhances chemotherapy and radiation responses and inhibits tumour growth in human gastric cancer xenografts. *Gut* 2005; **54**: 591-600

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DNA methylation biomarker candidates for early detection of colon cancer

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Abstract

Promoter CpG island hypermethylation of tumor suppressor genes is a common hallmark of all human cancers. Many researchers have been looking for potential epigenetic therapeutic targets in cancer using gene expression profiling with DNA microarray approaches. Our recent genome-wide platform of CpG island hypermethylation and gene expression in colorectal cancer (CRC)

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Conflicts of interest None

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cell lines revealed that *FBN2* and *TCERG1L* gene silencing is associated with DNA hypermethylation of a CpG island in the promoter region. In this study, promoter DNA hypermethylation of *FBN2* and *TCERG1L* in CRC occurs as an early and cancer-specific event in colorectal cancer. Both genes showed high frequency of methylation in colon cancer cell lines (>80% for both of genes), adenomas (77% for *FBN2*, 90% for *TCERG1L*, n=39), and carcinomas (86% for *FBN2*, 99% for *TCERG1L*, n=124). Bisulfite sequencing confirmed cancer-specific methylation of *FBN2* and *TCERG1L* of promoters in colon cancer cell line and cancers but not in normal colon. Methylation of *FBN2* and *TCERG1L* is accompanied by downregulation in cell lines and in primary tumors as described in the Oncomine™ website. Together, our results suggest that gene silencing of *FBN2* and *TCERG1L* is associated with promoter DNA hypermethylation in CRC tumors and may be excellent biomarkers for the early detection of CRC.

Keywords

DNA hypermethylation; Biomarker; Early detection; Colorectal Cancer (CRC); *FBN2*; *TCERGIL*

Introduction

Colorectal cancer (CRC) remains the second leading cause of cancer-related mortality in the USA [1]. Current screening modalities have resulted in only a modest decrease in mortality and have failed to achieve high public participation (<50%) [2, 3]. Strategies such as colonoscopy are invasive, whereas stool occult blood tests are of limited use because of the need for repeated measurements and interference by dietary components. The use of biomarkers, specifically epigenetic biomarkers, in serum, plasma, and stool are promising to close that gap between invasive but dependable tests and noninvasive but unreliable studies.

The past few years have seen an explosion of interest in the epigenetics of cancer. Epigenetic alterations have been widely recognized to play an important role in the development of cancer. Aberrant hypermethylation in the promoter regions of the tumor suppressor genes at CpG islands has been recognized as one of the hallmarks of cancer [4]. This hypermethylation of gene promoters is associated with downregulation of expression and the inactivation of critical tumor suppressor genes in human cancers [5]. Methylation of multiple genes in colorectal (CRC) cancer has been described including *hMLH1*, $p16^{INK4A}$, *APC*, *MGMT*, ,*sFRP1*, *GATA-4*and *GATA-5* [4, 6, 7]. More recently, DNA hypermethylation of *TFPI2* and *SOX17* genes have been detected in early stage of CRC patients [8].

We previously described a genome-wide expression array-based approach to identify the "DNA hypermethylome" in colon cancer estimated to contain about 500 hypermethylated genes per individual tumor [9]. Using this platform, we have identified a number of genes that are hypermethylated and underexpressed in colon cancers but not in normal tissues [9]. Furthermore, DNA methylation changes in cancer represent an attractive therapeutic target since epigenetic alterations are more readily reversible than genetic events [10]. However, the great strength of DNA methylation in the clinic promises to be in the area of molecular diagnostics and early detection.

Fibrillin 2 (*FBN2*), is an extracellular matrix protein. It is associated with elastic fibers in several tissues and is believed to serve as a ligand for alphavbeta3 integrin, the latter being a known morphogen. *FBN2* was first discovered to be expressed in the mesenchymal tissues and at the epitheliomesenchymal interface. It has also been demonstrated that the *FBN2* antisense oligodeoxynucleotide can induce dysmorphogenesis of the lung explants, suggesting that *FBN2* plays a key role in lung development [11]. Recently, the loss of *FBN2*

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expression due to promoter methylation was identified in pancreatic cancer cell lines by means of high-throughput microarray analysis [12].

Transcription elongation regulator 1-like (*TCERG1L*) gene located in chromosome 10, emerged as showing frequent cancer-specific methylation in our microarray-based approaches [9]. *TCERG1L* gene may have similar biological role with *TCERG1* (transcription elongation regulator 1), located on human chromosome 5q32. *TCERG1* (also known as TAF2S; TATA box-binding protein-associated factor 2S, CA150; transcription factor CA150) was first described as a transcriptional elongation regulator found in human immunodeficiency virus type 1 (HIV-1), tat-responsive HeLa nuclear fractions [13]. *TCERG1L* may have a function in the elongation-related factors in HeLa nuclear extracts [14] even though little else is known so far. Prior to this study, *TCERG1L* has not been described as being involved in cancer.

Here, we investigated promoter DNA methylation of *FBN2* and *TCERG1L* genes in CRC cell lines and primary tumors. We present data indicating that *FBN2* and *TCERG1L* genes are hypermethylated in most of CRC cell lines and primary tumors in a cancer-specific manner. Additionally, we demonstrate that methylation of *FBN2* and *TCERG1L* occurs in precancerous colon polyps suggesting that methylation of both of these genes is an early event in CRC. We suggest that *FBN2* and *TCERG1L* methylation could be a useful biomarker for noninvasive detection of precancerous and cancerous colon and rectal tumors.

Materials and methods

Cell culture and treatment

Colorectal cell lines (HCT116, RKO, HT29, SW480, DLD1, COLO 320,SW48, Lovo, Caco-2, andSW620) were obtained from ATCC (Manassas, VA, USA) and cultured in appropriate medium and under conditions described by ATCC, with media obtained from Invitrogen (Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). DKO cells (HCT116 cells with genetic disruption of *DNMT1* and *DNMT3b*) were cultured as previously described [15]. For demethylation studies, cultured cells were treated with 1 μM 5-aza-2′-deoxycytidine (DAC; Sigma, St. Louis, MO, USA) for 72 h with media changed every 24 h. Trichostatin A (TSA; Sigma, St. Louis, MO, USA) was obtained from Sigma and used to treat cells at concentration of 300 nM for 18 h. Mock drug treatments were performed in parallel with drug-free PBS.

Primer design

For expression studies using RT-PCR, we designed primers using the open access program Primer3 (http://frodo.wi.mit.edu/primer3). Primer sequences for methylation-specific polymerase chain reaction (MSP) and bisulfite sequencing analysis were designed using MSPPrimer (http://www.mspprimer.org) and their location in the *FBN2* and *TCERG1L* promoter is indicated in Fig. 3. All primer sequences are listed on Table 1.

Gene expression and methylation analyses

For MSP analysis, DNA was extracted following a standard phenol–chloroform extraction method. Bisulfite modification of genomic DNA was carried out using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA). We performed methylation analysis of the *FBN2* and *TCERG1L* promoter using MSP primer pairs covering the putative transcriptional start site in the 5′ CpG island with 1 μl of bisulfite-treated DNA as template and JumpStart Red Taq DNA Polymerase (Sigma, St. Louis, MO, USA) for amplification as previously described [16].

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Quantitative RT-PCR

Total RNA was extracted from all cell lines using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA), and was treated using the DNaseI protocol (QIAGEN, Valencia, CA, USA). One microgram total RNA was subjected to the Superscript II first-strand cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. For quantitative real-time analyses, the iQ™ SYBR Green Supermix kit (Bio-Rad) was used and the amplification conditions consisted of an initial 10-min denaturation step at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s and annealing and extension for 30 and 60 s, respectively. A Bio-Rad CFX384 real-time PCR System was used (Bio-Rad) and we used the comparative Ct method to compute relative expression values. We averaged expression of GAPDH as internal reference genes to normalized input cDNA. Also, we used normal colon and DKO cells (HCT116 cells with genetic disruption of *DNMT1* and *DNMT3b*) cDNA samples as positive controls for *FBN2* and *TCERG1L* gene expression.

Bisulfite sequencing analysis

One microgram of genomic DNA from each sample was bisulfite converted using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) following manufacturer's protocol. MSP primer sequences are provided in Table 1. The PCR amplicons were gel-purified and subcloned into pCRII-TOPO vector (Invitrogen, Carlsbad, CA, USA). At least ten clones were randomly selected and sequenced on an ABI3730xl DNA analyzer to ascertain the methylation patterns of each locus.

Primary human tissue samples

Primary tissue samples were obtained from the archives of the Department of Pathology, Johns Hopkins University with Institutional Review Board approval and Health Insurance Portability and Accountability Act compliance. We compiled data from 124 formalin-fixed and paraffin-embedded primary CRCs of tumor stages I to IV (stage I— n=33, stage II— n=37, stage III— n=36, stage IV— n=18; mean age, 65.2 years). We also examined 39 colorectal adenomas from patients without an associated invasive CRC (tubular— n=17, villous— n=22). We compared these samples to 20 normal colon controls of patients without any colorectal neoplasm (Table 2).

Results

Correlation between DNA promoter methylation and gene silencing of *FBN2* and *TCERG1L* in colon cancer cell lines

We previously developed an expression microarray technique to characterize the spectrum of hypermethylated genes in cancers [9]. Based on our previous study, some of the cancer-specific methylated genes detected in early stage of colon cancer could also function as diagnostic and prognostic biomarkers [8, 9]. *FBN2* and *TCERG1L* genes were identified in our hypermethylome approach and both have a dense CpG island in the promoter region upstream from the transcriptional start site (Fig. 3). To query these genes for methylation, we designed primers in promoter region of each gene for methylation-specific PCR (MSP) assays. Having confirmed our MSP primers and their monitoring of methylation density, we then tested both primers in ten different colon cancer cell lines (Fig. 1). We found complete methylation of *FBN2* promoter region in all ten colon cancer cell lines (100%, 10/10) (cell lines; HCT116, SW480, RKO, HT29, Caco-2, Lovo, COLO 320, COLO 205, DLD1, SW48, and SW620) (Fig. 1a). *TCERG1L* also showed complete methylation (80%, 8/10) in the majority of colon cancer cell lines except for Colo320 and Lovo (Fig. 1b). To ensure that methylation in these samples was associated with downregulation of gene expression, we then tested the expression of *FBN2* and *TCERG1L* using RT-PCR in a same panel of CRC

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cell lines with/without DAC and TSA treatment. Quantitative real-time PCR results confirmed re-expression of *FBN2* and *TCERG1L* genes after treatment with DAC, a demethylating agent, in most of CRC cell lines. Furthermore, we detected little or no expression of *FBN2* and *TCERG1L* in ten CRC lines, but all of these lines showed significantly increased expression of *FBN2 and TCERG1L* following DAC treatment (Fig. 1a,b). In the case of *TCERG1L*, even though it has basal expression in Lovo cells, its expression level increased after DAC treatment compared to basal expression. Interestingly, expression levels by RT-PCR of these two genes are not increased after treatment with TSA, a histone deacetylase inhibitor. Therefore, inactivation of *FBN2* and *TCERG1L* gene appears to be primarily regulated by promoter DNA hypermethylation in colon cancers.

Aberrant hypermethylation of the *FBN2* and *TCERG1L* gene in primary CRC tumors

Next, we expanded this study in a large cohort of CRC patient samples. One hundred twenty-four CRC samples (stages I–IV), 39 adenomas, and 20 normal patient samples were examined. Neither gene displayed methylation in normal colon samples (Fig. 2a). *FBN2* was found to be methylated in 90% (30/33) of stage I, 83% (31/37) of stage II, 91% (33/36) of stage III, and 72% (13/18) of stage IV colon cancers. *TCERG1L* was found to be methylated in 100% (33/33) of stage 1, 100% (37/37) of stage II, 97% (35/36) of stage 3, and 100% (18/18) of stage 4, CRC samples (Fig. 2b). This data indicates that *FBN2 and TCERG1L* gene methylation is cancer-specific. Moreover, we tested *FBN2* and *TCERG1L* promoter methylation in tubular and villous adenomas which are considered precancerous. Interestingly, *FBN2* methylation was observed in 71% of tubular adenomas (12 of 17), and 82% of villous adenomas (18 of 22). *TCERG1L* methylation was observed in 88% of tubular adenomas (15 of 17), and 91% of villous adenomas (20 of 22). This data strongly suggest that *FBN2* and *TCERG1L* hypermethylation occur as very early event during the progression of primary CRC. Therefore, detecting of DNA methylation for *FBN2* and *TCERG1L* genes could be useful as early detection biomarker in CRC.

We also confirmed DNA methylation status of *FBN2* and *TCERG1L* genes in a representative colon cancer cell line (HCT116) and primary CRC tumor sample by bisulfite sequencing analysis. Bisulfite sequencing of *FBN2* and *TCERG1L* gene promoter region showed dense DNA methylation in the colon cancer cell line HCT116 and CRC tumor sample and no methylation was detected in DKO cells and normal colonic tissues (Fig. 3). This data further supports our premise that *FBN2* and *TCERG1L* are methylated in a cancer-specific fashion in CRC.

FBN2 and *TCERG1L* gene expression is downregulated in CRC

Aberrant DNA hypermethylation has been well known to be associated with gene silencing in cancer. We observed *FBN2* and *TCERG1L* gene DNA hypermethylation is correlated with decreased gene expression and reactivated by DAC compare to basal expression level in colon cancer cell lines (Fig. 1a, b), suggesting that both of gene expression in colon cancer cell lines where methylation tightly correlated with lack of expression. However, since we have seen DNA hypermethylation of both genes in most of colon primary tissues, we wonder if these expression levels are correlated with DNA methylation status in colon primary tumors. Therefore, we utilized the gene expression microarray data previously published on Oncomine™ (Compendia Bioscience, Ann Arbor, MI, USA). The Oncomine™ database is a web-based data-mining platform aimed at facilitating gene discovery from genome-wide expression analyses in cancer [17]. We therefore queried the expression of *FBN2* and *TCERG1L* in colon primary tumors from several studies (Table 3). Very interestingly, we found that that both genes are significantly downregulated in six independent colon primary tumor datasets [18–21]. This information, in combination with

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our cell lines data, strongly suggests that *FBN2* and *TCERG1L* are methylated and downregulated in a cancer-specific fashion in CRC.

Discussion

Genome-wide approaches have contributed to our understanding of the molecular pathways driving tumorigenesis as well as providing useful new biomarkers for cancer risk assessment, early diagnosis, and prognosis [22]. Previous studies have confirmed that our microarray strategy is an effective approach to identify genes that are silenced by promoter hypermethylation in colon and breast cancer [9, 23, 24]. DNA methylation of gene promoters is among the earliest and most frequent oncologic alterations. A number of genes are commonly hypermethylated in CRC, however, genes methylated in early-stage tissues with high frequency are rare [9, 24, 25]. Previously, we have reported aberrant methylation of *TFPI2* and *SOX17* in CRC using our established array-based platform [26]. *TFPI2* gene promoter methylation has been identified in early stage of colon cancer and is highly sensitive in stool samples making it an excellent choice for noninvasive surveillance for CRC [8].

We have now discovered two additional genes which show cancer-specific methylation which are detected with high frequency in early stages of colorectal cancer formation. *FBN2* and *TCERG1L* are genes harboring cancer-specific promoter methylation in human colorectal cancer. Promoter DNA hypermethylation of *FBN2* and *TCERG1L* in CRC (86% for *FBN2*, 99% for *TCERG1L*, n=124, respectively) are frequent and cancer-specific events. Hypermethylation of *FBN2* has also been reported in pancreatic [12] and lung cancers [27–29], yet has not been described in CRC. *TCERG1L* has not previously been described in cancer. To our knowledge, this is the first description of *FBN2* and *TCERG1L* promoter methylation occurring as an early event in colon cancer progression.

Methylation biomarkers for the detection of an oncologic process requires that the DNA be obtained in a noninvasive fashion, either through blood, stool, or mucous. *TFPI2* has been shown to be sensitive and specific in stool samples. Plasma and serum biomarkers have also been shown to have relatively high specificity in CRC [30–32]. Therefore, testing our genes in stool, plasma, or serum, samples is necessary to confirm whether either gene could be useful biomarker for early detection in cancer in the near future. Additionally, other excellent early detection biomarkers have already been published by us and others [8, 33]. Any future study would need to test *FBN2* and *TCERG1L* in combination with *TFPI2* in colon cancer. It has been well known that methylation of CpG islands in gene promoter regions is associated with aberrant silencing of transcription and is a mechanism for inactivation of tumor suppressor genes (p16, APC, Rb) from many other studies [4, 25]. Recently, newly identified hypermethylated genes in cancer by genome-wide microarray approach showed its biological roles such as tumor-suppressive effect in cancer cells [8, 34]. Therefore, it could have a possibility that *FBN2* and *TCERG1L* might have tumor-suppressive effect in colon cancer but further study is need to define the functions of *FBN2* and *TCERG1L* in colon tumorigenesis.

In conclusion, *FBN2* and *TCERG1L* gene methylation is an early and frequent event in precancerous and cancerous lesions of the colon and rectum. Furthermore, this methylation is cancer-specific and was not detected in any normal colon samples. Finally, the methylation of *FBN2* and *TCERG1L* is associated with a phenotypic underexpression these gene products. These three factors warrant further examination of *FBN2* and *TCERG1L* in noninvasive studies for the early detection of colorectal cancer. We have reported for the first time that *FBN2* and *TCERG1L* promoter methylation is a feasible epigenetic marker for early detection of CRC and may be useful for CRC screening in the future.

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References

1. Jemal ASR, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics. CA Cancer J Clin. 2009; 59:225–249. [PubMed: 19474385]
2. Heresbach DMS, D'halluin PN, Bretagne JF, Branger B. Review in depth and meta-analysis of controlled trials on colorectal cancer screening by faecal occult blood test. Eur J Gastroenterol Hepatol. 2006; 18:427–433. [PubMed: 16538116]
3. Meissner HI, Breen N, Klabunde CN, Vernon SW. Patterns of colorectal cancer screening uptake among men and women in the United States. Cancer Epidemiol Biomarkers & Prevention. 2006; 15:389–394.
4. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med. 2003; 349:2042–2054. [PubMed: 14627790]
5. Jones PA, Baylin SB. The epigenomics of cancer. Cell. 2007; 128:683–692. [PubMed: 17320506]
6. Suzuki H, Watkins DN, Jair KW, Schuebel KE, Markowitz SD, Dong Chen W, et al. Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. Nat Genet. 2004; 36:417–422. [PubMed: 15034581]
7. Akiyama Y, Watkins N, Suzuki H, Jair KW, van Engeland M, Esteller M, et al. Gata-4 and gata-5 transcription factor genes and potential downstream antitumor target genes are epigenetically silenced in colorectal and gastric cancer. Mol Cell Biol. 2003; 23:8429–8439. [PubMed: 14612389]
8. Glockner SC, Dhir M, Yi JM, McGarvey KE, Van Neste L, Louwagie J, et al. Methylation of tfpi2 in stool DNA: a potential novel biomarker for the detection of colorectal cancer. Cancer Res. 2009; 69:4691–4699. [PubMed: 19435926]
9. Schuebel KE, Chen W, Cope L, Glöckner SC, Suzuki H, Yi JM, et al. Comparing the DNA hypermethylome with gene mutations in human colorectal cancer. PLoS Genetics. 2007; 3:1709–1723. [PubMed: 17892325]
10. Karpf ARJD. Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. Oncogene. 2002; 21:5496–5503. [PubMed: 12154410]
11. Yang Q, Ota K, Tian Y, Kumar A, Wada J, Kashihara N, et al. Cloning of rat fibrillin-2 cDNA and its role in branching morphogenesis of embryonic lung. Dev Biol. 1999; 212:229–242. [PubMed: 10419698]
12. Hagihara A, Miyamoto K, Furuta J, Hiraoka N, Wakazono K, Seki S, et al. Identification of 27 5′ CpG islands aberrantly methylated and 13 genes silenced in human pancreatic cancers. Oncogene. 2004; 23:8705–8710. [PubMed: 15467763]
13. Sune C, Hayashi T, Liu Y, Lane WS, Young RA, Garcia-Blanco MA. Ca150, a nuclear protein associated with the RNA polymerase ii holoenzyme, is involved in Tat-activated human immunodeficiency virus type 1 transcription. Mol Cell Biol. 1997; 17:6029–6039. [PubMed: 9315662]
14. Sanchez-Alvarez M, Goldstrohm AC, Garcia-Blanco MA, Sune C. Human transcription elongation factor ca150 localizes to splicing factor-rich nuclear speckles and assembles transcription and splicing components into complexes through its amino and carboxyl regions. Mol Cell Biol. 2006; 26:4998–5014. [PubMed: 16782886]
15. Rhee I, Bachman KE, Park BH, Jair KW, Yen RW, Schuebel KE, et al. DNMT1 and DNMT3B cooperate to silence genes in human cancer cells. Nature. 2002; 416:552–556. [PubMed: 11932749]
16. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A. 1996; 93:9821–9826. [PubMed: 8790415]

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17. Rhodes DRYJ, Shanker K, Deshpande N, Varambally R, Ghosh D, Barrette T, et al. Oncomine: a cancer microarray database and integrated data-mining platform. Neoplasia. 2004; 6:1–6. [PubMed: 15068665]
18. Kaiser SPY, Franklin JL, Halberg RB, Yu M, Jessen WJ, Freudenberg J, et al. Transcriptional recapitulation and subversion of embryonic colon development by mouse colon tumor models and human colon cancer. Genome Biol. 2007; 8:R131. [PubMed: 17615082]
19. Jorissen RN, Gibbs P, Christie M, Prakash S, Lipton L, Desai J, et al. Metastasis-associated gene expression changes predict poor outcomes in patients with dukes stage b and c colorectal cancer. Clin Cancer Res. 2009; 15:7642–7651. [PubMed: 19996206]
20. Smith JJ, Deane NG, Wu F, Merchant NB, Zhang B, Jiang A, et al. Experimentally derived metastasis gene expression profile predicts recurrence and death in patients with colon cancer. Gastroenterology. 2010; 138:958–968. [PubMed: 19914252]
21. Watanabe T, Kobunai T, Toda E, Yamamoto Y, Kanazawa T, Kazama Y, et al. Distal colorectal cancers with microsatellite instability (MSI) display distinct gene expression profiles that are different from proximal MSI cancers. Cancer Res. 2006; 66:9804–9808. [PubMed: 17047040]
22. Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. Nature. 2004; 429:457–463. [PubMed: 15164071]
23. Chan TAGS, Yi JM, Chen W, Van Neste L, Cope L, Herman JG, et al. Convergence of mutation and epigenetic alterations identifies common genes in cancer that predict for poor prognosis. PLoS Med. 2008; 5:0823–0838.
24. Yi JM, Dhir M, Van Neste L, Downing SR, Jeschke J, Glockner SC, et al. Genomic and epigenomic integration identifies a prognostic signature in colon cancer. Clin Cancer Res. 2011; 17:1535–1545. [PubMed: 21278247]
25. Esteller M. Epigenetic gene silencing in cancer: the DNA hypermethylome. Hum Mol Genet. 2007; 16:R50–R59. [PubMed: 17613547]
26. Zhang W, Glockner SC, Guo M, Machida EO, Wang DH, Easwaran H, et al. Epigenetic inactivation of the canonical Wnt antagonist SRY-box containing gene 17 in colorectal cancer. Cancer Research. 2008; 68:2764–2772. [PubMed: 18413743]
27. Tsunoda SSE, De Young NJ, Wang X, Tian ZQ, Liu JF, Jamieson GG, et al. Methylation of CLDN6, FBN2, RBP1, RBP4, TFPI2, and TMEFF2 in esophageal squamous cell carcinoma. Oncological Report. 2009; 21:1067–1073.
28. Cortese R, Hartmann O, Berlin K, Eckhardt F. Correlative gene expression and DNA methylation profiling in lung development nominate new biomarkers in lung cancer. Int J Biochem Cell Biol. 2008; 40:1494–1508. [PubMed: 18203646]
29. Chen H, Suzuki M, Nakamura Y, Ohira M, Ando S, Iida T, et al. Aberrant methylation of FBN2 in human non-small cell lung cancer. Lung Cancer. 2005; 50:43–49. [PubMed: 15951052]
30. Laird PW. The power and the promise of DNA methylation markers. Nat Rev Cancer. 2003; 3:253–266. [PubMed: 12671664]
31. Lofton-Day C, Model F, DeVos T, Tetzner R, Distler J, Schuster M, et al. DNA methylation biomarkers for blood-based colorectal cancer screening. Clin Chem. 2008; 54:414–423. [PubMed: 18089654]
32. Kim MSLJ, Sidransky D. DNA methylation markers in colorectal cancer. Cancer Metastasis Review. 2010; 29:181–206.
33. Melotte VLM, van den Bosch SM, Hellebrekers DM, de Hoon JP, Wouters KA, Daenen KL, et al. N-Myc downstream-regulated gene 4 (NDGR4): a candidate tumor suppressor gene and potential biomarker for colorectal cancer. J Natl Canc Inst. 2009; 101:916–927.
34. Melotte V, Lentjes MHFM, van den Bosch SM, Hellebrekers DMEI, de Hoon JPJ, Wouters KAD, et al. N-Myc downstreamregulated gene 4 (NDRG4): a candidate tumor suppressor gene and potential biomarker for colorectal cancer. J National Cancer Institute. 2009; 101:916–927.

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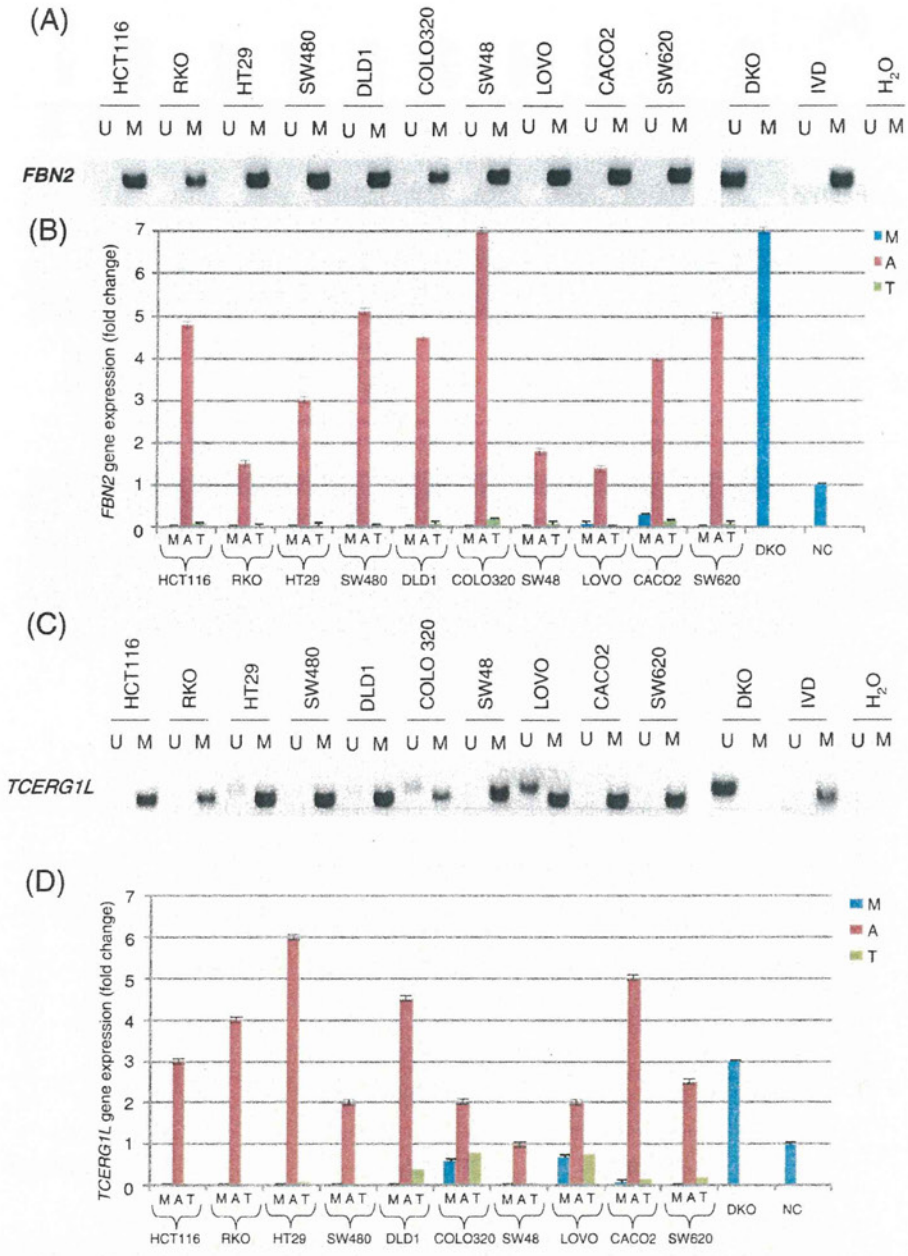

Fig. 1.

Epigenetic inactivation of *FBN2* and *TCERG1L* in colon cancer cell lines. Methylation analysis of (**a**) *FBN2* and (**b**) *TCERG1L* gene promoter (Upper panel) and real-time RT-PCR results for (**c**) *FBN2* and (**d**) *TCERG1L* gene in colon cancer cell lines after DAC and TSA treatment with mock in each cell lines. *GAPDH* was used as control for equal amplification. PCR products recognize unmethylated (U) and methylated (M). DKO cells [15] were used for unmethylated control. IVD = *in-vitro* methylated control, ddH_2O=water control containing no DNA. M, A, and T indicate mock, DAC treated cells, Trichostatin A (TSA) treated cells, respectively

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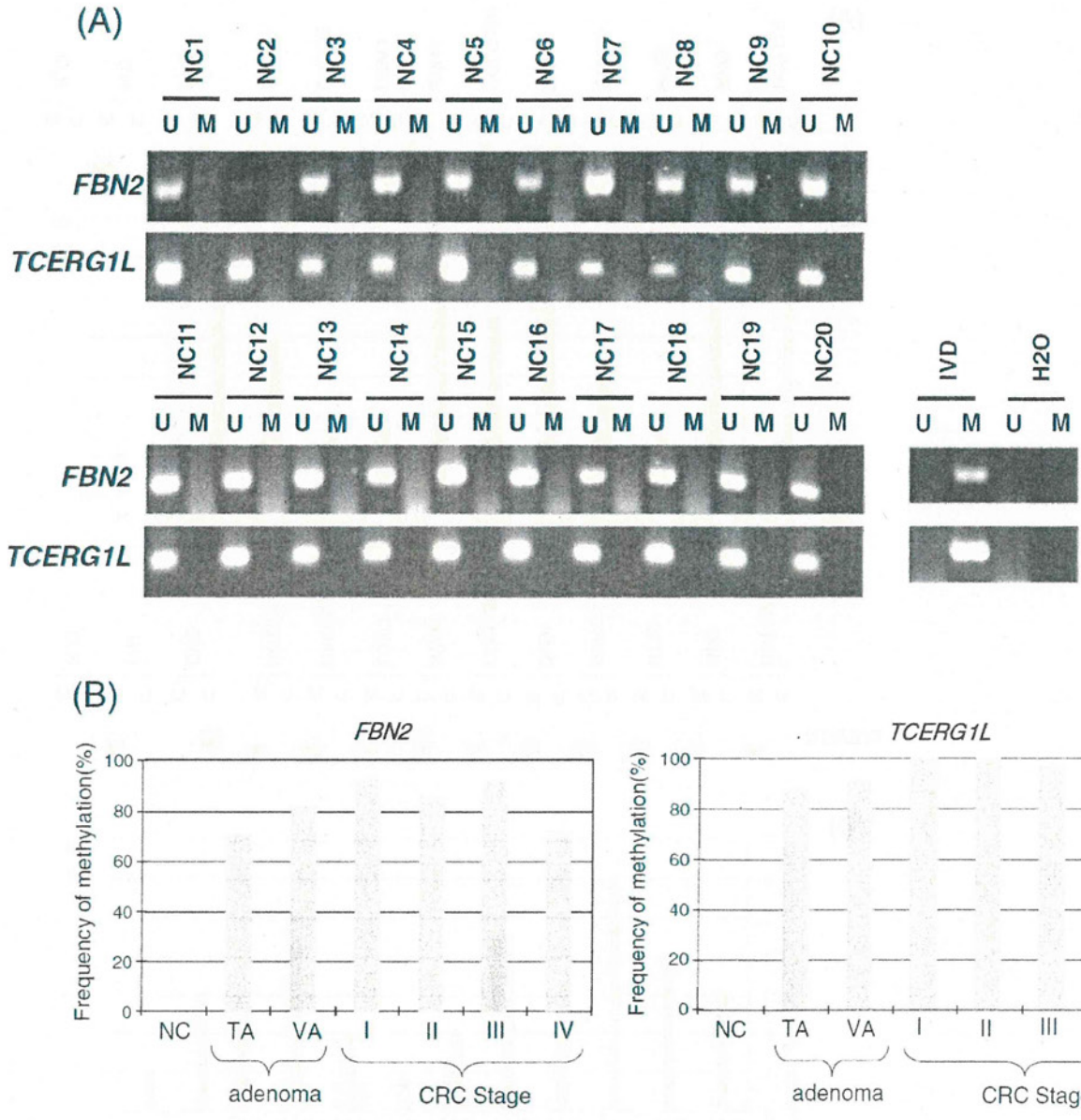

Fig. 2.

Epigenetic inactivation of *FBN2* and *TCERG1L* in colon primary tissues versus normal colon tissues. (**a**) normal colon tissues (**b**) primary CRC tissues. M = methylation signal; U = unmethylated signal. IVD = in vitro methylated DNA. ddH_2O=water control adding no DNA. Numbers represent normal colon (NA1-20). NC indicates normal colon. Bar graph showing MSP results of all primary samples (stage I-IV: n=124) and adenomas (tubular adenoma (TA), villous adenoma (VA), n=39)

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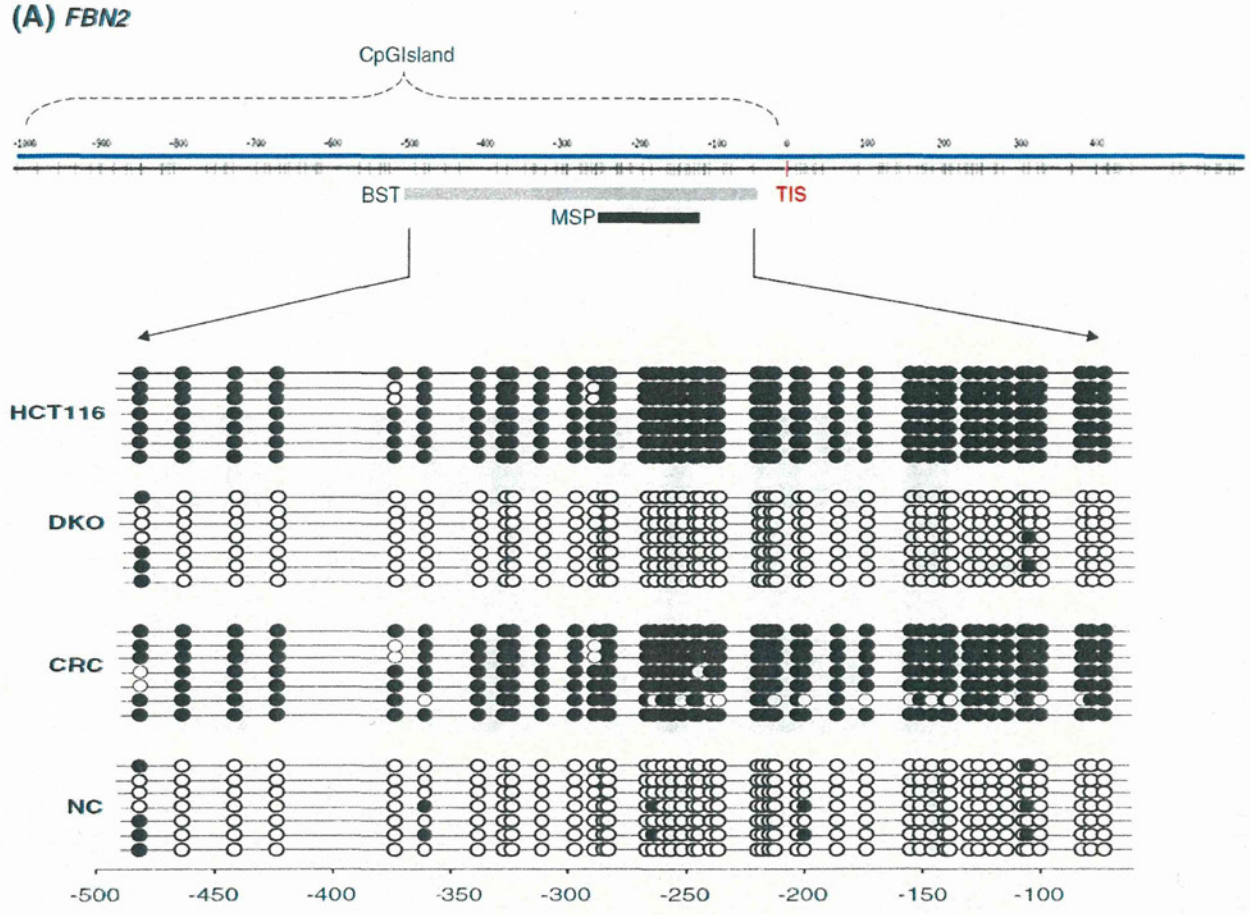

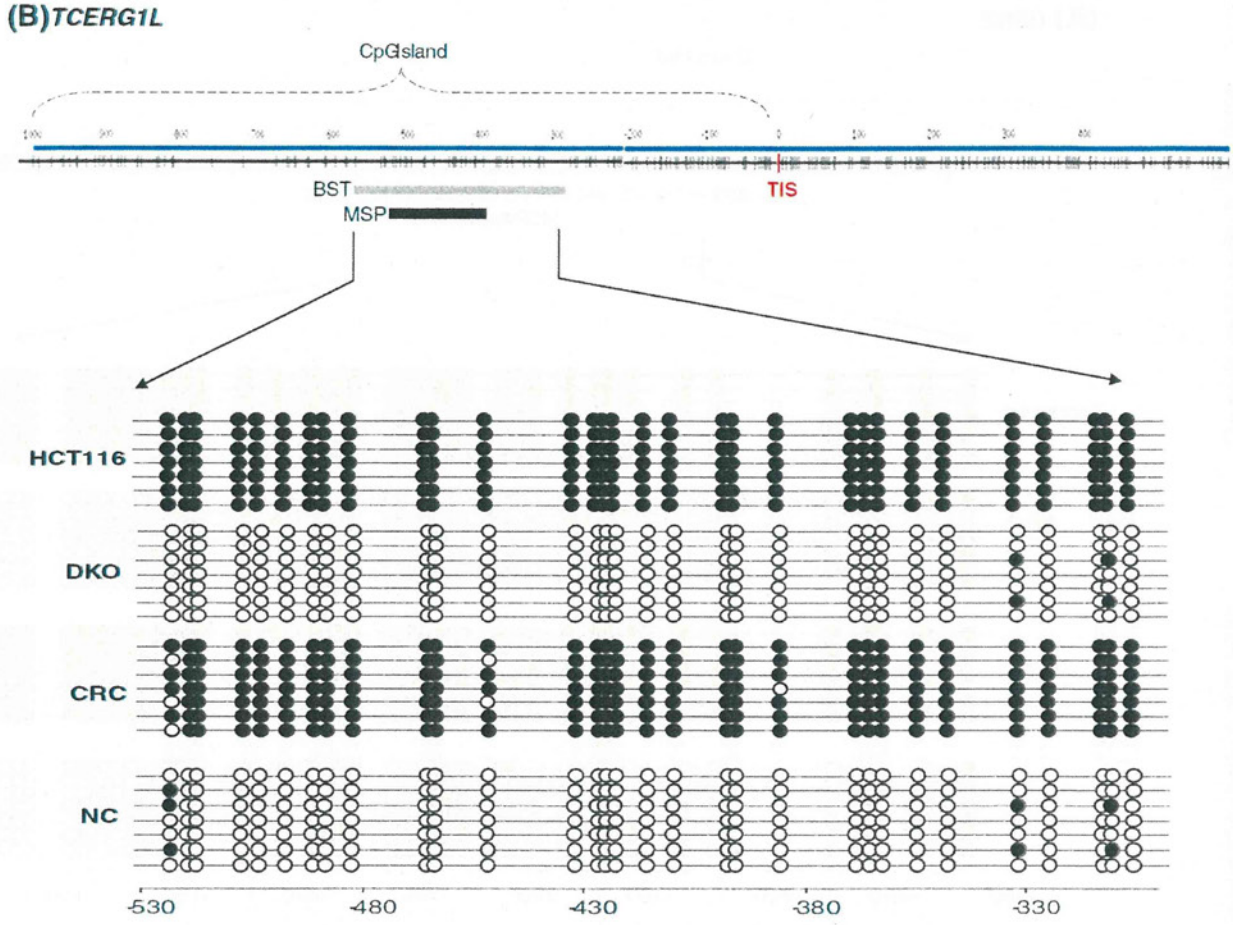

Fig. 3.

Representative Bisulfite sequencing results of *FBN2* (**a**) and *TCERG1L* (**b**) in HCT116, DKO, a primary CRC (CRC20), and normal colon (NC10). Open and filled circles represent unmethylated and methylated CpG sites, respectively, and each row represents a single clone. Location of CpG sites (*FBN2*: upstream region from - 475 to –64; *TCERG1L*: upstream region from –556 to –275) relative to the transcription start sites (TIS) of Exon 1 is shown Fig. 3. Both genes bisulfate sequencing region include MSP analysis amplicons

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Table 1

Primers for MSP, RT-PCR, and bisulfite sequencing analysis

Gene		S (5′–3′)	AS(5′–3′)
FBN2	Meth	GGGTTTTTAAAATTTTCGCGTCGC	CTACGAAACCGAACGAAAATACG
	Unmeth	GTTTTGTTGGGTTTTTAAAATTTTTGTGTTGTG	AAATAACAACTACAAAACCAAACAAAAATACA
	RT	AGGGATCCTTCATTTGCCAC	GCCCGGGGTATTTACACACT
	BST	CTTCCAACCCYACCTTC	GTTTTTAGAAGAAGAGGAGGG
TCERG1L	Meth	GGTCGTTTGCGTCGGATTC	CTACCCAACGCGAAACTAAAAACG
	Unmeth	TTTGGGGTTGTTTGTGTTGGATTTG	CATATCCCACTACCCAACACAAAACTAAAAACA
	RT	TGCTCTTGCAACCCCTATTG	TGAGGTGCTAACACCACCGT
	BST	AATTTGTTTGGTTTATTTGTGTAATAGAAAT	CTAATAACCTCTAACCCTCTAA

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Table 2

Baseline characteristics of CRC patients in this study

Characteristics	*N* (%)
CRC (*n*=124)	
Age (years)	
Median	65.2
Sex	
Male	68 (55%)
Female	56 (45%)
Race	
White	84 (68%)
Black	33 (27%)
Asian	2 (1%)
Other	5 (4%)
Grade	
Well-differentiated	39 (31%)
Moderately differentiated	63 (51%)
Poorly differentiated	22 (18%)
Location	
Right	71 (58%)
Left	51 (41%)
Right and left	2 (1%)
Stage	
I	33 (27%)
II	37 (30%)
III	36 (29%)
IV	18 (14%)
Adenomas (*n*=39)	
Tubular	17 (44%)
Villous	22 (56%)
Normal colon (*n*=20)	20