COMMENTARY

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Quantitative validation of *GJC1* promoter hypermethylation in benign and malignant colorectal tumors

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Abstract

We have previously shown that the gap junction protein γ 1 (*GJC1*) gene, encoding the connexin-45 protein, is inactivated by promoter hypermethylation in colorectal cancer. This was confirmed in a recent *Endocrine-Related Cancer* publication analyzing a limited number of samples. The aim of this study was to analyze *GJC1* in a larger clinical cohort ($n=485$) and to assess whether or not the promoter hypermethylation was associated with clinical or pathological features. The methylation of *GJC1* was confirmed to be tumor specific and was observed in 33% of colorectal cancers and 12% of adenomas. The methylation was strongly associated with *BRAF* mutations ($P=5.64\times10^{-13}$) as well as with proximal tumor location ($P=1.42\times10^{-3}$), features compatible with a CpG island methylator phenotype.

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Introduction

Recently, we investigated the promoter DNA methylation status, assessed by qualitative methylation-specific PCR (MSP), of the connexin gene family members in colorectal cancer (Sirnes *et al.* 2011). The gap junction protein γ 1 (*GJC1*), encoding the connexin-45 protein, was found to be frequently hypermethylated in primary colorectal carcinomas and unmethylated in normal mucosa samples. Furthermore, methylation of *GJC1* was associated with reduced gene expression, and treatment with the demethylating agent 5-aza-2′deoxycytidine led to re-expression of *GJC1* in colon cancer cell lines.

GJC1 methylation analysis in a large clinical sample series

In a recent publication in *Endocrine-Related Cancer*, Mori *et al.* (2011) confirmed that *GJC1* is a potential biomarker for discriminating colorectal cancer patients from controls. The authors used methylated CpG island amplification coupled with microarray analysis and a well-designed data analysis pipeline to identify 169 candidate loci for cancer-specific hypermethylation. *GJC1* was among the 14 genes successfully tested with quantitative MSP (qMSP) in a small series of carcinomas ($n=51$), adenomas ($n=9$), and normal mucosa ($n=54$). In that study, the authors pointed out that *GJC1*, as well as the other identified loci, 'merit a large-scale independent validation study' (Mori *et al.* 2011). In this study, we have used qMSP (forward primer: TATTCGAGCGTTACGTGTCGC; reverse primer: CGCCTACGCACTACGCG; probe: 6FAM-TCGTTTTCGGGTCGCG-MGB) to analyze test and validation sets of malignant and benign tumors as well as normal mucosa samples, counting altogether 485 samples (Tables 1 and 2). The percentile of the highest percent methylated reference (PMR = 3) value across the normal mucosa samples in the test set was used as a fixed threshold for scoring methylation-positive samples in both the test and validation series. Promoter hypermethylation was identified in 12% of the adenomas, 33% of the carcinomas, 2% of the normal mucosa samples taken in distance form the carcinoma, and in none of the normal mucosa

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Table 1 Frequency and distribution of promoter methylation levels (PMR values) sample material measured by quantitative methylation-specific PCR (qMSP)

	Normal mucosa		Normal mucosa from cancer patients		Adenomas		Carcinomas		
GJC1	*n* (%)	Median methylated samples (IQR)	*n* (%)	Median methylated samples (IQR)	*n* (%)	Median methylated samples (IQR)	*n* (%)	Median methylated samples (IQR)	*P* value[a]
Test set	0/51 (0%)	– (–)	– (–)	– (–)	7/61 (11%)	14.7 (37.7)	20/64 (31%)	22.3 (14.9)	1.9×10^{-4}
Validation set	0/56 (0%)	– (–)	2/105 (2%)	7.7 (5.4)	6/43 (14%)	13.3 (32.9)	35/105 (33%)	31.0 (27.2)	2.0×10^{-10}
Combined (test and validation)	0/107 (0%)	– (–)	2/105 (2%)	7.7 (5.4)	13/104 (12%)	14.7 (36.5)	55/169 (33%)	26.3 (22.0)	6.6×10^{-14}

IQR, interquartile range (75th percentile–25th percentile); *n*, number of positive cases; PMR, percent methylated reference.
[a]Kruskal–Wallis test.

samples obtained from cancer-free individuals (Table 1 and Fig. 1).

In recent years, the CpG island methylator phenotype (CIMP) has been suggested to be an important pathway in the development of colorectal cancer (Toyota *et al*. 1999, Weisenberger *et al*. 2006). CIMP-positive tumors are characterized by concordant hypermethylation in several CpG loci and include the majority of sporadic colorectal cancers with a microsatellite unstable (MSI) phenotype. Hence, CIMP tumors are associated with many of the features typical of MSI tumors, such as proximal location and *BRAF* mutation (Weisenberger *et al*. 2006, Shen *et al*. 2007) and have also been associated with improved patient prognosis (Ogino *et al*. 2009). When comparing the promoter methylation status of *GJC1* with genetic and clinicopathological features, we discovered that *GJC1* methylation was more common among MSI (29/45, 64%) primary colorectal carcinomas than among MSS tumors (26/124, 21%; $P=2.44\times10^{-7}$). In line with the CIMP concept, the *GJC1* promoter methylation was in addition significantly associated with proximal tumor location ($P=1.42\times10^{-3}$; also reported by Mori *et al*. (2011)), and the presence of *BRAF* mutations in exon 15 ($P=5.64\times10^{-13}$). The mean PMR value in cancers from female patients (12.96) was significantly higher than that seen in male patients (7.07; Student's *t*-test, $P=0.026$), but no significant difference was seen among normal mucosa samples. In addition, no significant association was seen between DNA methylation and tumor stage or age of the patients.

Receiver operating characteristic (ROC) curves are well suited to determine weather potential biomarkers can discriminate patient samples from normal controls. In concordance with (Mori *et al*. 2011), we observe an area under the ROC curve (AUC) of 0.67 (95% confidence interval (CI): 0.61–0.73; $P=1.6\times10^{-6}$) for discriminating colorectal carcinomas from normal mucosa samples. Not surprisingly, and probably due to the larger sample series analyzed here (104 adenomas versus nine), the AUC value for discriminating benign tumors from controls was better than previously reported (0.61. 95% CI 0.53–0.68; $P=7.3\times10^{-3}$; Table 3). Although somewhat improved, these values are still low, and in a diagnostic setting *GJC1* would be outperformed by a number of colorectal tumor biomarkers, including *VSX2* (AUC 0.93) and the other promising biomarkers identified in the same genome-wide search (Mori *et al*. 2011). The authors

Table 2 Overview of clinical samples included in this study

	Normal mucosa		Normal mucosa from cancer patients	Adenoma		Carcinoma	
Series	Test (*n*=51)	Validation (*n*=56)	Validation (*n*=105)	Test (*n*=61)	Validation (*n*=43)	Test (*n*=64)	Validation (*n*=105)
Median patient age (range)	55 (22–86)	67 (63–72)	71 (29–93)	67 (62–72)	58 (50–64)	71 (33–92)	71 (29–93)
Description of cohort	Deceased colorectal cancer-free individuals, collected at the Institute of Forensic Medicine, University of Oslo, Norway	Colorectal adenoma and carcinoma-free individuals, confirmed by sigmoidoscopy (Thiis-Evensen *et al*. 1999)	Matching the carcinoma validation series	Individuals attending a population-based screening study (Thiis-Evensen *et al*. 1999)	Individuals attending a second population-based screening study (Bretthauer *et al*. 2002)	Obtained from a prospective series collected between 1987 and 1989 (Meling *et al*. 1991)	Derived from a prospective series from the Department of Surgery at Oslo University Hospital, Aker Hospital

According to National legislation all samples belong to approved research biobanks and approvals are given by the Regional Ethics Committee (S-09282c2009/4958 biobank 2781;S95151).

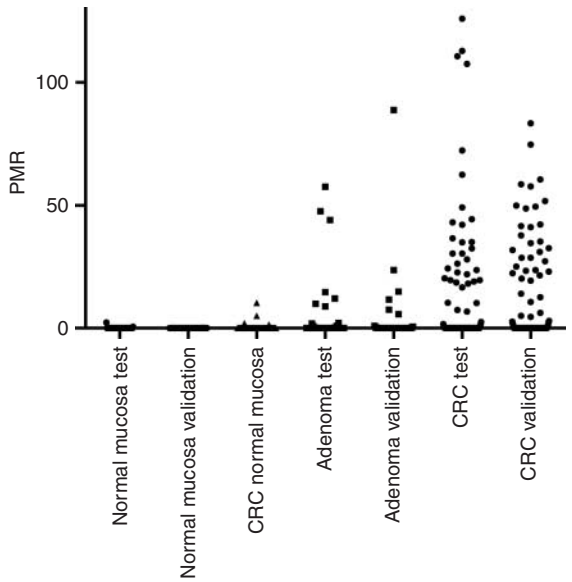

Figure 1 Dot plot of percent methylated reference (PMR) values of *GJC1* in test and validation sets of normal mucosa, adenomas, and colorectal cancer. CRC, colorectal cancer.

point out that although the combination of these markers did not improve the diagnostic accuracy compared with *VSX2* alone, this might be achieved by including existing colorectal tumor biomarkers. The recently identified *SPG20* (AUC 0.95; Lind *et al*. 2011*b*) could be an alternative as well as *CNRIP1* (AUC 0.98) and *MAL* (AUC 0.96; Lind *et al*. 2008, 2011*a*).

Conclusion

The results by Mori *et al*. (2011), as well as the quantitative results presented here for a rather large clinical sample series validate our initial findings and pinpoint promoter hypermethylation of *GJC1* as a tumor-specific event (Sirnes *et al*. 2011). In addition to colorectal carcinomas, *GJC1* hypermethylation was seen among a small subset of adenomas, indicating that the reduction or loss of GJC1 (connexin-45) protein expression can occur early in the colorectal tumorigenesis. Finally, the promoter methylation was restricted to a specific subgroup of colorectal tumors with CIMP-like features, suggesting a role for connexin-45 in the development of colorectal cancer.

Table 3 Receiver operating characteristic (ROC) curve analysis of *GJC1*

Samples	**AUC** (95% CI)	***P* value**
CRC ($n=169$)	0.67 (0.61–0.73)	1.6×10^{-6}
Stage I and II CRC ($n=101$)	0.69 (0.62–0.76)	2.0×10^{-6}
Stage III and IV CRC ($n=68$)	0.64 (0.55–0.73)	1.5×10^{-3}
Adenoma ($n=104$)	0.61 (0.53–0.68)	7.3×10^{-3}
CRC normal ($n=105$)	0.51 (0.43–0.59)	7.8×10^{-1}

ROC curve analysis for the discrimination of tissues from normal mucosa (from colorectal cancer-free individuals). Data are shown for test and validation series combined (lines 1 and 4), and stratified according to tumor stage (lines 2 and 3). AUC, area under the curve; CI, confidence interval; CRC, colorectal cancer.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

Bretthauer M, Gondal G, Larsen K, Carlsen E, Eide TJ, Grotmol T, Skovlund E, Tveit KM, Vatn MH & Hoff G 2002 Design, organization and management of a controlled population screening study for detection of colorectal neoplasia: attendance rates in the NORCCAP Study (Norwegian Colorectal Cancer Prevention). *Scandinavian Journal of Gastroenterology* **37** 568–573. (doi:10.1080/00365520252903125)

Lind GE, Ahlquist T, Kolberg M, Berg M, Eknaes M, Alonso MA, Kallioniemi A, Meling GI, Skotheim RI, Rognum TO *et al.* 2008 Hypermethylated MAL gene – a silent marker of early colon tumorigenesis. *Journal of Translational Medicine* **6** 13. (doi:10.1186/1479-5876-6-13)

Lind GE, Danielsen SA, Ahlquist T, Merok MA, Andresen K, Skotheim RI, Hektoen M, Rognum TO, Meling GI, Hoff G *et al.* 2011*a* Identification of an epigenetic biomarker panel with high sensitivity and specificity for colorectal cancer and adenomas. *Molecular Cancer* **10** 85. (doi:10.1186/1476-4598-10-85)

Lind GE, Raiborg C, Danielsen SA, Rognum TO, Thiis-Evensen E, Hoff G, Nesbakken A, Stenmark H & Lothe RA 2011*b* SPG20, a novel biomarker for early detection of colorectal cancer, encodes a regulator of cytokinesis. *Oncogene* **30** 3967–3978. (doi:10.1038/onc.2011.109)

Meling GI, Lothe RA, Børresen AL, Hauge S, Graue C, Clausen OP & Rognum TO 1991 Genetic alterations within the retinoblastoma locus in colorectal carcinomas. Relation to DNA ploidy pattern studied by flow cytometric analysis. *British Journal of Cancer* **64** 475–480. (doi:10.1038/bjc.1991.334)

Mori Y, Olaru AV, Cheng Y, Agarwal R, Yang J, Luvsanjav D, Yu W, Selaru FM, Hutfless S, Lazarev M *et al.* 2011 Novel candidate colorectal cancer biomarkers identified by methylation microarray-based scanning. *Endocrine-Related Cancer* **18** 465–478. (doi:10.1530/ERC-11-0083)

Ogino S, Nosho K, Kirkner GJ, Kawasaki T, Meyerhardt JA, Loda M, Giovannucci EL & Fuchs CS 2009 CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. *Gut* **58** 90–96. (doi:10.1136/gut.2008.155473)

Shen L, Toyota M, Kondo Y, Lin E, Zhang L, Guo Y, Hernandez NS, Chen X, Ahmed S, Konishi K *et al.* 2007 Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. *PNAS* **104** 18654–18659. (doi:10.1073/pnas.0704652104)

Sirnes S, Honne H, Ahmed D, Danielsen SA, Rognum TO, Meling GI, Leithe E, Rivedal E, Lothe RA & Lind GE 2011 DNA methylation analyses of the connexin gene family reveal silencing of GJC1 (connexin45) by promoter hypermethylation in colorectal cancer. *Epigenetics* **6** 602–609. (doi:10.4161/epi.6.5.15237)

Thiis-Evensen E, Hoff GS, Sauar J, Langmark F, Majak BM & Vatn MH 1999 Population-based surveillance by colonoscopy: effect on the incidence of colorectal cancer. Telemark Polyp Study I. *Scandinavian Journal of Gastroenterology* **34** 414–420. (doi:10.1080/003655299750026443)

Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB & Issa JP 1999 CpG island methylator phenotype in colorectal cancer. *PNAS* **96** 8681–8686. (doi:10.1073/pnas.96.15.8681)

Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH, Widschwendter M, Weener D, Buchanan D *et al.* 2006 CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nature Genetics* **38** 787–793. (doi:10.1038/ng1834)

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