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=5.64×10^{-13}) as well as with proximal tumor location (P=1.42×10^{-3}), features compatible with a CpG island methylator phenotype.

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: TATTCGAG-CGTTACGTGTCGC; reverse primer: CGCCTACGC-ACTACCGG; probe: 6FAM-TCGTTTTCGGGTCG-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 samples.
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 tumors from controls was better than previously reported (0.61; 95% CI 0.53–0.68; P = 7.3 × 10⁻³; 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

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 × 10⁻⁵) 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 × 10⁻³; 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
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. 2011b) could be an alternative as well as CNRIP1 (AUC 0.98) and MAL (AUC 0.96; Lind et al. 2008, 2011a).

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

<table>
<thead>
<tr>
<th>Samples</th>
<th>AUC (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mucosa</td>
<td>0.67 (0.61–0.73)</td>
<td>1.6×10⁻⁶</td>
</tr>
<tr>
<td>Adenoma</td>
<td>0.69 (0.62–0.76)</td>
<td>2.0×10⁻⁶</td>
</tr>
<tr>
<td>CRC test</td>
<td>0.64 (0.55–0.73)</td>
<td>1.5×10⁻³</td>
</tr>
<tr>
<td>CRC validation</td>
<td>0.61 (0.53–0.68)</td>
<td>7.3×10⁻³</td>
</tr>
<tr>
<td>CRC normal</td>
<td>0.51 (0.43–0.59)</td>
<td>7.8×10⁻¹</td>
</tr>
</tbody>
</table>

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.

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.

Figure 2 Receiver operating characteristic (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.

Figure 3 Scatter plot of percent methylated reference (PMR) values of MAL in test and validation sets of normal mucosa, adenomas, and colorectal cancer. CRC, colorectal cancer.

Figure 4 Scatter plot of percent methylated reference (PMR) values of GJC1 in test and validation sets of normal mucosa, adenomas, and colorectal cancer. CRC, colorectal cancer.

Figure 5 Scatter plot of percent methylated reference (PMR) values of VSX2 in test and validation sets of normal mucosa, adenomas, and colorectal cancer. CRC, colorectal cancer.

Figure 6 Scatter plot of percent methylated reference (PMR) values of SPG20 in test and validation sets of normal mucosa, adenomas, and colorectal cancer. CRC, colorectal cancer.

Figure 7 Scatter plot of percent methylated reference (PMR) values of CNRIP1 in test and validation sets of normal mucosa, adenomas, and colorectal cancer. CRC, colorectal cancer.

Figure 8 Scatter plot of percent methylated reference (PMR) values of MAL in test and validation sets of normal mucosa, adenomas, and colorectal cancer. CRC, colorectal cancer.

Figure 9 Scatter plot of percent methylated reference (PMR) values of GJC1 in test and validation sets of normal mucosa, adenomas, and colorectal cancer. CRC, colorectal cancer.

Figure 10 Scatter plot of percent methylated reference (PMR) values of VSX2 in test and validation sets of normal mucosa, adenomas, and colorectal cancer. CRC, colorectal cancer.

Figure 11 Scatter plot of percent methylated reference (PMR) values of SPG20 in test and validation sets of normal mucosa, adenomas, and colorectal cancer. CRC, colorectal cancer.

Figure 12 Scatter plot of percent methylated reference (PMR) values of CNRIP1 in test and validation sets of normal mucosa, adenomas, and colorectal cancer. CRC, colorectal cancer.

Figure 13 Scatter plot of percent methylated reference (PMR) values of MAL in test and validation sets of normal mucosa, adenomas, and colorectal cancer. CRC, colorectal cancer.

Figure 14 Scatter plot of percent methylated reference (PMR) values of GJC1 in test and validation sets of normal mucosa, adenomas, and colorectal cancer. CRC, colorectal cancer.

Figure 15 Scatter plot of percent methylated reference (PMR) values of VSX2 in test and validation sets of normal mucosa, adenomas, and colorectal cancer. CRC, colorectal cancer.

Figure 16 Scatter plot of percent methylated reference (PMR) values of SPG20 in test and validation sets of normal mucosa, adenomas, and colorectal cancer. CRC, colorectal cancer.

Figure 17 Scatter plot of percent methylated reference (PMR) values of CNRIP1 in test and validation sets of normal mucosa, adenomas, and colorectal cancer. CRC, colorectal cancer.

Figure 18 Scatter plot of percent methylated reference (PMR) values of MAL in test and validation sets of normal mucosa, adenomas, and colorectal cancer. CRC, colorectal cancer.

Figure 19 Scatter plot of percent methylated reference (PMR) values of GJC1 in test and validation sets of normal mucosa, adenomas, and colorectal cancer. CRC, colorectal cancer.

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D Ahmed et al.: GJC1 promoter hypermethylation in CRC


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