Abstract

**Purpose:** Little information is available on genetic and epigenetic changes in duodenal adenocarcinomas. The purpose was to identify possible subsets of duodenal adenocarcinomas based on microsatellite instability (MSI), DNA methylation, mutations in the \( \text{KRAS} \) and \( \text{BRAF} \) genes, clinicopathologic features, and prognosis.

**Experimental Design:** Demographics, tumor characteristics, and survival were available for 99 duodenal adenocarcinoma patients. Testing for \( \text{KRAS} \) and \( \text{BRAF} \) mutations, MSI, \( \text{MLH1} \) methylation, and CpG island methylator phenotype (CIMP) status was conducted. A Cox proportional hazard model was built to predict survival.

**Results:** CIMP\(^+\) was detected in 27 of 99 (27.3%) duodenal adenocarcinomas and was associated with MSI (\( P = 0.011 \)) and \( \text{MLH1} \) methylation (\( P < 0.001 \)), but not with \( \text{KRAS} \) mutations (\( P = 0.114 \)), as compared with CIMP/C0 tumors. No \( \text{BRAF} \) V600E mutation was detected. Among the CIMP\(^+\) tumors, 15 (55.6%) were CIMP\(^+\)/\( \text{MLH1} \)-unmethylated (\( \text{MLH1} \)-U). Kaplan–Meier analysis showed that tumors classified by CIMP, CIMP/\( \text{MLH1} \) methylation status, or CIMP/MSI status could predict overall survival (OS; \( P = 0.047, 0.002, \) and 0.002, respectively), whereas CIMP/\( \text{MLH1} \) methylation status could also predict time-to-recurrence (TTR; \( P = 0.016 \)). In multivariate analysis, CIMP/\( \text{MLH1} \) methylation status showed a significant prognostic value in both OS (\( P < 0.001 \)) and TTR (\( P = 0.023 \)). Patients with CIMP\(^+\)/\( \text{MLH1} \)-U tumors had the worst OS and TTR.

**Conclusions:** Our results showed existence of CIMP in duodenal adenocarcinomas. The combination of CIMP\(^+\)/\( \text{MLH1} \)-U seems to be independently associated with poor prognosis in patients with duodenal adenocarcinomas. This study also suggests that \( \text{BRAF} \) mutations are not involved in duodenal tumorigenesis, MSI, or CIMP development.
levels were set at plus 2 SDs of the average methylation levels observed in normal duodenal mucosa controls. Samples were considered CIMP$^+$ if at least 3 of the 5 studied genes were methylated (6).

Conventional methylation-specific PCR (MSP; ref. 19) was carried out to validate the CIMP status by using a panel of 20 cancer-specific genes/loci (6, 7, 9, 20–27), as well as the 5 genes used for MethyLight. Gene and locus names and primers are shown in Supplementary Table S1. Methylation index was calculated as total number of genes and loci methylated/total number of genes and loci analyzed (28).

Immunohistochemistry

Immunohistochemical analysis for MLH1 expression was carried out. In brief, FFPE tissues were sectioned at 6 μm and stained with antibody to MLH1 (BD PharMingen). Tumor cells with absent nuclear staining were interpreted to have an absence of protein expression. Intact nuclear staining of adjacent nonneoplastic epithelium served as an internal positive control.

Statistical methods

Differences in categorical variables between study groups were analyzed using $\chi^2$ test for homogeneity of Fisher exact test. To compare continuous variables, the Student $t$ test was used when variances were equal. The Mann–Whitney $U$ test was used when variances were unequal. Correlation between MethyLight and MSP results were analyzed by Fisher exact test. All hypotheses tests were 2-sided, and results were considered statistically significant for $P$ values less than 0.05.

The main outcome of this study was overall survival (OS), which was defined as the time from surgery to death resulting from any cause. In addition, time-to-recurrence (TTR) was defined as the time from surgery to recurrence, in

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Abbreviations: U, unmethylated; M, methylated.

*CIMP$^+$ versus CIMP$^-$, $\chi^2$ test unless indicated otherwise.

$^a$Fisher exact test.
which patients without evidence of recurrence were censored for TTR at last follow-up. Survival was estimated by using the Kaplan–Meier method and log-rank statistics to test for differences between survival curves for various prognostic factors. Cox proportional hazard models were used to calculate HR with corresponding 95% confidence interval (CI) of recurrence or death according to molecular features (i.e., CIMP/MLH1 methylation, CIMP, MLH1 methylation, or MSI status), adjusted for age, sex, stage, tumor differentiation, chemotherapy and/or radiotherapy, and KRAS mutation status. All calculations were done using SPSS 16.0 software (SPSS Inc.).

**Results**

**Clinicopathologic characteristics by CIMP**

DNA extraction and CIMP testing by MethyLight were successful in all 99 patients. Twenty-seven patients (27.3%) of the 99 patients tested were CIMP⁺ (Fig. 1, Table 1).

To further determine whether the 5-gene signature accurately classifies patients as CIMP⁺ and validate the CIMP status as characterized by MethyLight, we determined the methylation of an additional panel of 20 genes/loci using conventional MSP in a group of samples. These genes were selected because they have either been previously used to identify CIMP or showed frequent methylation in various cancers, including duodenal cancers (6, 7, 9, 20–27). Aberrant methylation was significantly more frequent in tumors characterized as CIMP⁺, using the 5-gene signature, with a methylation index of 0.67 (average 13.4 genes methylated of 20 genes examined) compared with a methylation index of 0.14 (average 2.8 genes methylated of 20 genes examined) in tumors characterized as CIMP⁻, showing a marked difference (P < 0.001; Supplementary Fig. S1). In addition, comparison between the 5-gene methylation status using MethyLight technology and MSP analysis revealed significant correlations (κ = 0.583–0.813). These results suggested that the 5-gene signature was successful in identifying a CIMP⁺ subset of tumors.

Median age at diagnosis of duodenal cancer was 66.0 years (65.4 ± 13.4; mean ± SD). Comparison of the CIMP⁺ and CIMP⁻ subgroups showed that there were no differences in gender, age, tumor differentiation, extent of resection, and undergoing chemotherapy/radiotherapy between the 2 groups (Table 1). Although not statistically significant, a trend toward association between stage and CIMP status was observed (P = 0.067). The CIMP⁺ group had more stage I tumors than the CIMP⁻ group (18.5% vs. 4.2%).

**MSI, CIMP, and MLH1 methylation**

Among the 99 duodenal cancer patients, 20 (20.2%) displayed MSI-H, 14 (14.1%) MSI-L, and 65 (65.7%) MSS status. In this study, MSI-L and MSS tumors were grouped together and henceforth are referred to as MSS. Among the 27 (27.3%) patients showing CIMP⁺, 10 (37.0%) were MSI as well (Fig. 1, Table 1). A statistically significant correlation between MSI and CIMP status was observed (P = 0.011, Table 1).

MLH1 methylation was detected in 14 (14.1%) patients and 12 (85.7%) were also CIMP⁺. Further associations showed that 8 (57.1%) of these were CIMP⁺/MSI, 4 (28.6%) were CIMP⁺/MSS, 2 (14.3%) were CIMP⁻/MSI, and none showed CIMP⁻/MSS (Fig. 1). There were strong associations between MLH1 methylation and both MSI and CIMP⁺ (P < 0.001, all). Distributions of MSI, CIMP, and MLH1 methylation in all patients are shown in Supplementary Fig. S2.

**Immunohistochemical analysis of MLH1 in tumors**

Immunohistochemical analysis of MLH1 expression was carried out on selected MLH1 unmethylated (MLH1-U) and MLH1 methylated (MLH1-M) tumors. All tested MLH1-M tumors (including 4 MSS/MLH1-M tumors) had negative or low protein expression level (Fig. 2).

**Frequency and associations of tumor mutations**

Mutation analysis was successfully conducted in all 99 tumors and matched normal duodenal tissue specimens for KRAS and BRAF. KRAS mutations were prevalent in 32.3% (32 of 99) of patients and the characteristics of patients with KRAS mutations are shown in Supplementary Table S2. The most prevalent KRAS mutations were GGT > GAT (G12D) and GGT > GTT (G12V) within codon 12, and GCC > GAC (G13D) within codon 13. All mutations seem to be somatic because the same alterations were not detected in the corresponding normal tissues. Twenty-five of 32 cases (78.1%) with KRAS mutations occurred in

Figure 1. Heat map showing relationship of specific gene methylation, KRAS mutations, MSI status, and categorization as CIMP⁺ and CIMP⁻ in duodenal adenocarcinomas. IVD, in vitro methylated DNA (M.SssI-reference); PMR, percent of methylated reference.
tumors exhibiting methylation in at least 1 of the 6 study genes (OR, 3.08, 1.17 to 8.08; \( P = 0.020 \)). However, KRAS mutations were not associated with CIMP (\( P = 0.114 \), Table 1), as compared with wild-type tumors. No BRAF V600E mutation was found in any tumor or corresponding normal duodenal tissue.

Survival analysis by CIMP and MSI

Median follow-up of patients was 36.9 months for OS analysis and 30.5 months for TTR analysis. Kaplan–Meier survival curves were generated according to clinicopathologic and molecular characteristics. The median OS for the entire group was 53.7 months, with 5- and 10-year OS of 49% and 35%, respectively. Age, stage, and MSI status were the 3 important predictors of OS with older age and late stage conferring worse OS, whereas MSI was associated with improved OS, as expected (log-rank \( P < 0.05 \), all; Supplementary Fig. S3). CIMP\(^+\) was significantly associated with shorter OS (log-rank \( P = 0.047 \); Fig. 3A). The median OS time was 33.9 months in patients with CIMP\(^+\) tumors (5- and 10-year OS of 36% and 27%, respectively) compared with 90.8 months in patients with CIMP\(^-\) tumors (5- and 10-year OS of 53% and 47%; Supplementary Table S3). CIMP alone was, however, not a predictor for TTR (log-rank \( P = 0.608 \); Fig. 3B). The median TTR time for the CIMP\(^-\) group was 123.4 months and had not been reached for the CIMP\(^+\) group. Age, stage, differentiation, and chemotherapy and/or radiotherapy were predictors of TTR with young age, late stage, poor differentiation, and undergoing chemotherapy and/or radiotherapy conferring worse TTR (log-rank \( P < 0.05 \), all; Supplementary Fig. S4).

Tumors were further classified by CIMP and MLH1 methylation status into CIMP\(^-\)/MLH1-U (\( n = 70 \)), CIMP\(^-\)/MLH1-M (\( n = 2 \)), CIMP\(^+\)/MLH1-U (\( n = 15 \)), and CIMP\(^+\)/MLH1-M (\( n = 12 \)) groups. There were significant differences both in OS (log-rank \( P = 0.002 \); Fig. 4A) and TTR (log-rank \( P = 0.016 \); Fig. 4B) in the groups classified by CIMP/MLH1 methylation status. CIMP\(^+\)/MLH1-U group had the shortest OS and TTR, whereas CIMP \(/MLH1\)-M group had the longest OS and TTR. CIMP\(^-\)/MLH1-M group consisted of 2 patients with a remarkable recurrence-free follow-up of 85.8 and 144.9 months at the conclusion of the study, respectively.

Tumors were also categorized by CIMP and MSI status into CIMP\(^-\)/MSS (\( n = 62 \)), CIMP\(^-\)/MSI (\( n = 10 \)), CIMP\(^+\)/MSS (\( n = 17 \)) and CIMP\(^+\)/MSI (\( n = 10 \)) groups. In the groups classified by CIMP/MSI status, there was significant difference in OS (log-rank \( P = 0.002 \); Fig. 4C), but not in TTR (log-rank \( P = 0.196 \); Fig. 4D) with CIMP\(^+\)/MSS group having the worst OS.
Multivariate analysis of outcome predictors

A Cox proportional hazards model for multivariate analysis, including CIMP/MLH1 methylation status, age, sex, stage, tumor differentiation, chemotherapy and/or radiotherapy, and KRAS mutation status in relation to OS and TTR was conducted (Table 2). Only CIMP/MLH1 methylation status \((P < 0.001)\), age \((P = 0.002)\), and stage \((P < 0.001)\) remained statistically significant as predictors of OS. CIMP/MLH1 methylation status \((P = 0.023)\), stage \((P = 0.020)\), along with tumor differentiation \((P = 0.034)\) were also associated with risk of recurrence and independently predicted TTR.

The influence of CIMP, MLH1 methylation, or MSI on OS and TTR, independent of the clinicopathologic and molecular variables were separately assessed (Supplementary Table S4). In multivariate analyses, CIMP by itself only showed a trend toward correlation with both OS \((P = 0.081)\) and TTR \((P = 0.176)\). MLH1 methylation status...
Methylation in Duodenal Adenocarcinomas

was independently associated with OS ($P = 0.021$), but not TTR ($P = 0.070$). MSI independently correlated with both OS ($P = 0.003$) and TTR ($P = 0.018$).

Discussion

The CIMP was first characterized in human CRC by our group as cancer-specific CpG island hypermethylation of a subset of genes in a subset of tumors (7). Weisenberger and colleagues confirmed and further characterized CRC CIMP using MethyLight technology (6). Since then CIMP has been shown in multiple other malignancies, including gastric (29), pancreatic (20), lung (21), oral (22), breast (30), and small intestinal cancers (31), as well as neuroblastoma (32), malignant melanoma (33), and glioma (33). In this study, we analyzed a large cohort of patients with duodenal adenocarcinomas and showed that CIMP existed in 27.3% of the tumors.

There is no consensus with regard to the best markers for defining CIMP in duodenal cancer. Fang and colleagues compared the CIMP-associated loci from breast cancer, colon cancer, and glioma, and found that the CIMP signature was shared by multiple human malignancies (30). By using CIMP-associated loci in CRC, previous studies have successfully identified CIMP tumors in duodenal cancers (9, 31). In this study, CIMP was defined by a panel of 5 markers proposed and validated by Weisenberger and colleagues (6). This 5-gene signature used has been shown to be highly accurate and the most cost-effective screening method for CIMP status in CRC (6). The question therefore arises as to whether this panel of markers would also be applicable in duodenal cancers. Ideally, it would be helpful to use a whole epigenomic approach to define CIMP in cancers. However, this is not feasible given the rarity of duodenal adenocarcinomas and lack of appropriate fresh tissue samples to conduct this analysis. To confirm whether the 5-gene signature truly differentiates a CIMP group, we screened a panel of 20 commonly used markers for CIMP. Importantly, duodenal tumors that were identified as CIMP by the 5-gene signature were concordant with those positive on the large-scale screen. Our results showed that this 5-gene signature correlated with CIMP and accurately define CIMP in duodenal adenocarcinomas.

It has been established that KRAS and BRAF mutations have a number of downstream effectors that can activate or repress genes, and which may then contribute to patterning the epigenome (34). In our data, KRAS gene mutation was associated with tumors that had at least one gene methylated, and this is in accordance with the evidence of

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<th>P</th>
<th>HR (95% CI)</th>
<th>P</th>
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<td>1.00 (Referent)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>61</td>
<td>1.42 (0.79–2.57)</td>
<td>0.246</td>
<td>1.10 (0.55–2.20)</td>
<td>0.778</td>
<td>3.67 (1.49–9.03)</td>
<td>0.005</td>
<td>1.87 (0.71–4.92)</td>
<td>0.203</td>
</tr>
<tr>
<td>KRAS mutations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>67</td>
<td>1.00 (Referent)</td>
<td>1.00 (Referent)</td>
<td>1.00 (Referent)</td>
<td>1.00 (Referent)</td>
<td></td>
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</tr>
<tr>
<td>Present</td>
<td>32</td>
<td>1.09 (0.61–1.95)</td>
<td>0.772</td>
<td>0.64 (0.34–1.20)</td>
<td>0.160</td>
<td>1.02 (0.50–2.09)</td>
<td>0.960</td>
<td>0.51 (0.22–1.17)</td>
<td>0.111</td>
</tr>
</tbody>
</table>

Abbreviations: MLH1-M, MLH1-methylated; MLH1-U, MLH1-unmethylated.
induction of the ras oncogenic pathway may result in DNA methylation (35). Yet it seems that KRAS mutations alone do not dictate duodenal cancer CIMP status because KRAS mutations were not associated with CIMP as compared with wild-type tumors.

Moreover in CRC, CIMP status has been associated with mutations of the BRAF gene and has been felt to be mutually exclusive to KRAS mutations (6). In fact, we did not detect any mutations in codon 600 of the BRAF gene. This is in keeping with Blaker and colleagues who described only one mutation [a 3 bp (GAT) deletion at codon 603/604] in a panel of 21 adenocarcinomas of the small intestine (36). It is possible that other mutations exist outside codon 600 of the BRAF gene and would have been detected if we had screened the whole of the BRAF gene; however, most BRAF mutations in human cancers are within codon 600 (10, 37). These data lead us to conclude that BRAF mutations are not critically involved in duodenal tumorigenesis, MSI, or CIMP development.

It was reported that CIMP⁺ CRCs have a distinct clinicopathologic and molecular features, such as associations with proximal tumor location, female sex, poor differentiation and mucinous tumor histology, MSI as a consequence of hypermethylation of the MLH1 gene, and high BRAF mutation rate (6). In our study, we were unable to find any association of CIMP with gender or tumor differentiation, but not surprisingly, we found a strong correlation between CIMP and both MLH1 methylation and MSI in duodenal cancers. Interestingly, we also found that only half of CIMP⁺ tumors showed MLH1 methylation and a small number of MLH1 methylated tumors were CIMP⁻. Immunohistochemical analysis of MLH1 expression in those MLH1-M tumors also validated the methylation. These observations are consistent with a stochastic process of cancer methylation and a gradually increasing probability of MLH1 methylation (38). On the basis of a limited number of cases, we found that CIMP⁺ duodenal cancers had a relatively earlier stage when compared with CIMP⁻ tumors, though this was not statistically significant. The result indicates that CIMP development is an early event in some cases of duodenal cancer.

Several studies have investigated the relationship between CIMP status and survival in various malignancies. However, these results are inconsistent (30, 39–46). The association of better clinical outcome with CIMP⁺ tumors has been reported in CRC, gliomas, and breast cancer. Poor prognosis with CIMP⁺ tumors has also been reported across CRC (44), esophageal cancer (45), gastric cancer (46), myelodysplastic syndromes (43), neuroblastomas (41), and leukemia (42). The discrepancy of these observations might be because of different methylation markers of CIMP panels, methodologies for methylation detection, patient populations, distribution of tumor stages and differentiation, terms of follow-up, and other factors associated with prognosis being included (such as chemotherapy and/or radiotherapy).

The prognostic significance of CIMP status in duodenal cancer has not previously been described. We identified a patient population that was CIMP⁺ and found that this inversely correlated with survival. Stratification of CIMP by MSI status was predictive of OS but not TTR. However, stratification of CIMP by MLH1 methylation status further enhances the ability to predict OS as well as predict TTR in CIMP⁺ patients on multivariate analysis. CIMP⁺/MLH1⁻U patients had a poorer outcome than individuals with CIMP⁻/MLH1⁻U tumors and indeed compared with all other individuals in the study. Interestingly, there were only 2 patients with CIMP⁻ but MLH1⁻methylated tumors, both of them did extremely well with long-term follow-up. This is particularly surprising because one of the tumors was poorly differentiated, the other was moderately differentiated, and both were advanced stage (stage III). Most importantly, in 27 CIMP⁺ tumors, there were 17 MSS and 10 MSI tumors. Even though multivariate analysis showed that MSI was an independent predictor of OS and TTR, stratification of CIMP⁺ tumors by MSI could not clearly define 2 subtypes. On the contrary, MLH1 methylation status segregated the CIMP⁺ tumors into 15 MLH1⁻U tumors and 12 MLH1⁻M tumors. Both subtypes behave differently with significantly different OS and TTR, which indicate that CIMP⁺ tumors may follow 2 different pathways. The significant survival difference between the groups classified by CIMP/MLH1 methylation status might imply a clue to the complexity of CIMP development. It suggests that not only oncogenic pathways but also epigenetic pathways themselves jointly affect CIMP in a pattern of reciprocal causation.

An important limitation of our study is the lack of statistical power because of a low number of patients in some subgroups might obscure more subtle relations. The analysis in a larger cohort of duodenal adenocarcinomas is needed to validate our findings.

In conclusion, our data suggests that CIMP does exist in duodenal adenocarcinomas and it may assist in the prognostic classification of these patients. Stratification of CIMP by MLH1 methylation status enhances the ability to predict OS as well as predict TTR. Patients with CIMP⁺ duodenal adenocarcinomas, especially those with CIMP⁺ tumors in absence of MLH1 methylation, may need more intensive surveillance and subtype-specific adjuvant therapy strategies. We did not detect any BRAF V600E mutation, which suggests that BRAF mutations are not critically involved in duodenal tumorigenesis, MSI, or CIMP development. These results give new insight into the genetic and epigenetic pathways of duodenal adenocarcinoma and show the need for further understanding of these unique tumors.

Disclosure of Potential Conflicts of Interest
S.B. Baylin has commercial grant funding and serves on the advisory board for MDx Health Inc. and BioNumerik Pharmaceuticals Inc. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Fu, E.P. Pappou, A.A. Guzzetta, R. Kwak, P. Dave, R. Morgan, C.A. Iacobuzio-Donahue, N. Ahuja
CIMP and MLH1 Methylation in Duodenal Adenocarcinomas

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Acknowledgments

The authors thank Kathy Bender and Ricky Moore for administrative support and Sharon Metzger-Gaud, Theresa San Lorenzo-Caswell, and the Johns Hopkins Cancer Registry for assistance with the primary cancer databases.

Grant Support

This work was supported by NIH grant CA140599 and CA127141, National Natural Science Foundation of China grant 81000898, and American College of Surgeons - Society of University Surgeons.

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Received March 3, 2012; revised June 28, 2012; accepted July 4, 2012; published OnlineFirst July 23, 2012.

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References

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