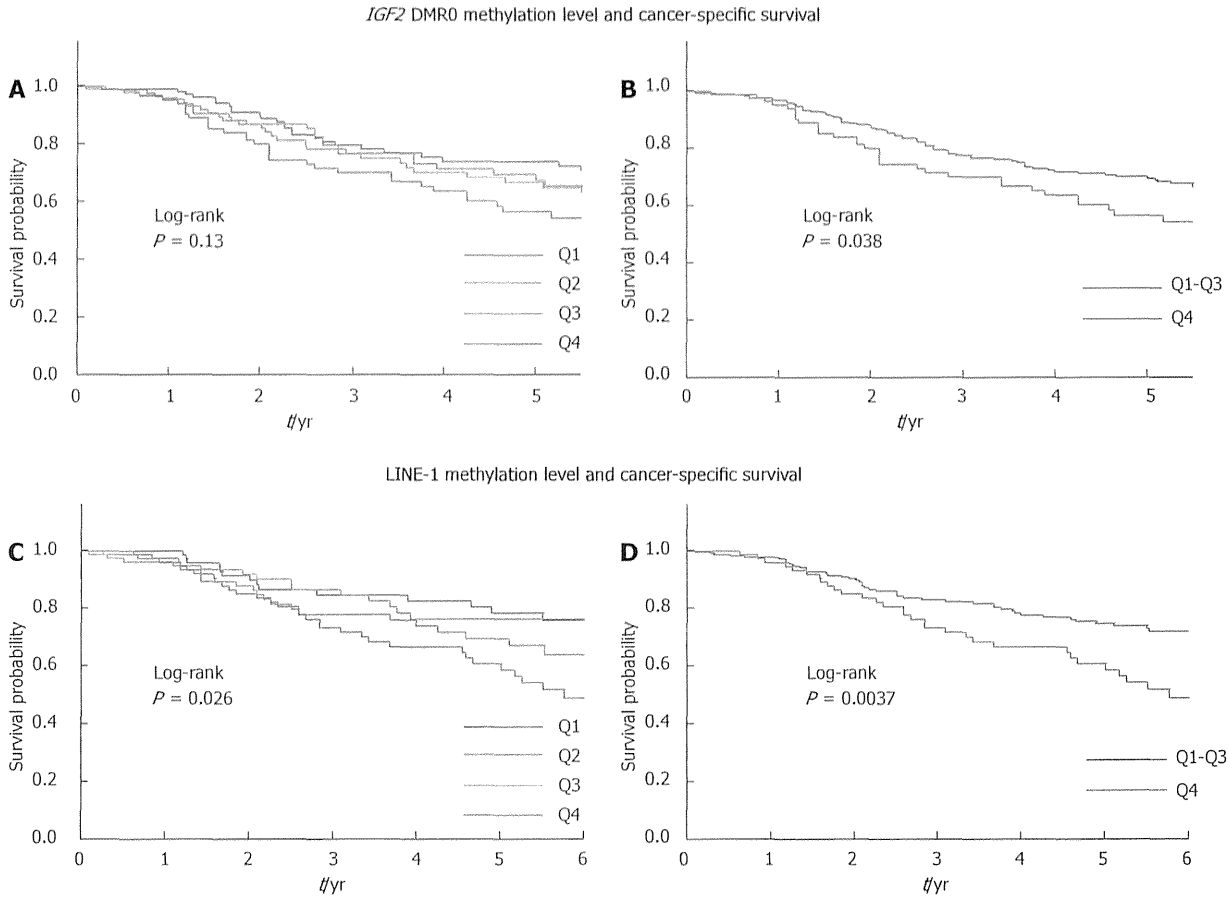


**Figure 3 Kaplan-Meier survival curves for colorectal cancer according to the *IGF2* differentially methylated region 0 and long interspersed nucleotide element-1 methylation levels in metastatic colorectal cancers.** A: Patients with *Insulin-like growth factor 2* (*IGF2*) differentially methylated region (DMR)0 hypomethylation had a slightly higher mortality rate than those with *IGF2* DMR0 hypermethylation, but this difference was not significant (log-rank test: $P = 0.13$); B: *IGF2* DMR0 hypomethylation (Q4 cases) was significantly associated with unfavorable cancer-specific survival (log-rank test: $P = 0.038$); C: Significantly higher mortality was observed in patients with long interspersed nucleotide element-1 (LINE-1) hypomethylation compared with those with LINE-1 hypermethylation (log-rank test: $P = 0.026$); D: LINE-1 hypomethylation (Q4 cases) was significantly associated with unfavorable cancer-specific survival (log-rank test: $P = 0.0037$).

censored patients was 3.3 years. Kaplan-Meier analysis was performed using categorical variables (Q1, Q2, Q3, and Q4). Slightly but insignificantly higher mortality was observed in patients with *IGF2* DMR0 hypomethylation compared with those without hypomethylation in terms of cancer-specific survival (log-rank test: $P = 0.13$) (Figure 3A). In another Kaplan-Meier analysis, Q4 cases were defined as the "hypomethylated group" and the Q1, Q2, and Q3 cases were combined into a "non-hypomethylated group"; the hypomethylated group (log-rank test: $P = 0.038$) was found to have significantly higher mortality (Figure 3B). Similar results were observed in terms of overall survival (log-rank test: $P = 0.040$) (data not shown).

### *LINE-1 methylation level and CRC patient survival*

The LINE-1 methylation level in CRC was also divided into quartiles (Q1 $\geq$ 58.7, Q2 54.8-58.6, Q3 50.8-54.7, and Q4 $\leq$ 50.7). A significantly higher mortality rate was observed among Q4 cases (log-rank test: $P = 0.0037$) in the Kaplan-Meier analysis (Figure 3C, D).

### *Association of histological type and IGF2 DMR0 and LINE-1 methylation levels as well as other molecular features of serrated lesions and non-serrated adenomas*

Table 2 shows the clinicopathological and molecular features of serrated lesions and non-serrated adenomas. No significant difference was observed between SSAs (69.0 ± 10.8) with cytological dysplasia and SSAs without (73.1 ± 12.3) in *IGF2* DMR0 methylation levels ($P = 0.32$). In contrast, MSI-high was more frequently ($P < 0.0001$) found in SSAs with cytological dysplasia [40% (4/10)] than in SSAs [0.8% (1/120)]. With regard to the LINE-1 methylation level, no significant difference was observed between the methylation level and histological type in serrated lesions and non-serrated adenomas ($P = 0.59$).

Mutations of *BRAF*, *KRAS*, and *PIK3CA* were detected in 49%, 19%, and 0.9% of HPs, 87%, 2.5%, and 0% of SSAs, 69%, 17%, and 0% of TSAs and 2.6%, 19%, and 1.3% of non-serrated adenomas, respectively (Table 2).

**Table 2 Clinical and molecular features of serrated lesions and non-serrated adenomas (tubular or tubulovillous adenoma) according to histological type *n* (%)**

| Clinical or molecular feature | Total *n* | Histological type | | | | | | *P* value |
|---|---|---|---|---|---|---|---|---|
| | | Serrated lesion | | | | Non-serrated adenoma | | |
| | | HP | SSA without cytological dysplasia | SSA with cytological dysplasia | TSA without high-grade dysplasia (HGD) | Tubular adenoma without HGD | Tubulovillous adenoma without HGD | |
| All cases | 416 | 115 | 120 | 10 | 91 | 77 | 3 | |
| Sex | | | | | | | | |
| Male | 263 (63) | 78 (68) | 72 (60) | 5 (50) | 55 (60) | 50 (65) | 3 (100) | 0.36 |
| Female | 153 (37) | 37 (32) | 48 (40) | 5 (50) | 36 (40) | 27 (35) | 0 (0) | |
| Age (mean ± SD) | 60.3 ± 11.8 | 57.5 ± 12.1 | 57.2 ± 11.6 | 74.1 ± 4.7 | 60.9 ± 12.3 | 66.6 ± 11.4 | 66.0 ± 8.9 | < 0.0001 |
| Tumor size (mm) (mean ± SD) | 10.5 ± 5.4 | 9.3 ± 3.7 | 11.6 ± 5.4 | 12.3 ± 6.4 | 9.7 ± 4.7 | 10.9 ± 7.2 | 15.7 ± 13.2 | 0.0069 |
| Tumor location | | | | | | | | |
| Rectum | 42 (10) | 15 (13) | 0 (0) | 0 (0) | 16 (18) | 10 (14) | 1 (33) | < 0.0001 |
| Distal colon | 127 (31) | 44 (39) | 17 (14) | 1 (10) | 39 (44) | 25 (34) | 1 (33) | |
| Proximal colon | 239 (59) | 54 (48) | 103 (86) | 9 (90) | 34 (38) | 38 (52) | 1 (33) | |
| *BRAF* mutation | | | | | | | | |
| Wild-type | 183 (44) | 59 (51) | 16 (13) | 2 (20) | 28 (31) | 75 (97) | 3 (100) | < 0.0001 |
| Mutant | 231 (55) | 56 (49) | 104 (87) | 8 (80) | 61 (69) | 2 (2.6) | 0 (0) | |
| *KRAS* mutation | | | | | | | | |
| Wild-type | 357 (87) | 92 (81) | 117 (98) | 10 (100) | 74 (83) | 62 (81) | 2 (67) | < 0.0001 |
| Mutant | 55 (13) | 21 (19) | 3 (2.5) | 0 (0) | 15 (17) | 15 (19) | 1 (33) | |
| *PIK3CA* mutation | | | | | | | | |
| Wild-type | 406 (99) | 113 (99) | 117 (100) | 10 (100) | 89 (100) | 74 (99) | 3 (100) | 0.67 |
| Mutant | 2 (0.5) | 1 (0.9) | 0 (0) | 0 (0) | 0 (0) | 1 (1.3) | 0 (0) | |
| MSI status | | | | | | | | |
| MSS/MSI-low | 408 (98) | 113 (98) | 119 (99) | 6 (60) | 90 (99) | 77 (100) | 3 (100) | 0.0004 |
| MSI-high | 8 (1.9) | 2 (1.7) | 1 (0.8) | 4 (40) | 1 (1.1) | 0 (0) | 0 (0) | |
| *IGF2* DMR0 methylation level (mean ± SD) | 64.5 ± 17.2 | 61.9 ± 20.5 | 73.1 ± 12.3 | 69.0 ± 10.8 | 61.6 ± 19.6 | 58.9 ± 16.1 | 61.0 ± 7.1 | < 0.0001 |
| LINE-1 methylation level (mean ± SD) | 58.7 ± 5.0 | 58.6 ± 3.4 | 58.1 ± 5.4 | 58.3 ± 8.4 | 58.8 ± 4.7 | 59.4 ± 6.0 | 60.9 ± 1.4 | 0.59 |

Percentage indicates the proportion of patients of each histological type who met the criteria for a specific clinical or molecular feature. *P* values were calculated by analysis of variance for age, tumor size, methylation levels of *IGF2* DMR0 and LINE-1 and by $\chi^2$ or Fisher's exact test for all other variables. The *P* value for significance was adjusted by Bonferroni correction to 0.0050 (= 0.05/10). HGD: High-grade dysplasia; HP: Hyperplastic polyp; MSI: Microsatellite instability; MSS: Microsatellite stable; SSA: Sessile serrated adenoma; TSA: Traditional serrated adenoma; *IGF2*: *Insulin-like growth factor* 2.

### *IGF2* DMR0 and LINE-1 hypomethylation in TSAs and non-serrated adenomas with high-grade dysplasia

Tables 3 and 4 show the clinicopathological and molecular features of the TSAs (with or without HGD), non-serrated adenomas (with or without HGD), and CRCs (stages I -Ⅳ). The *IGF2* DMR0 methylation levels in TSAs with HGD (50.2 ± 18.7) were significantly lower than those in TSAs without (61.6 ± 19.6, $P = 0.038$) (Table 3). With regard to LINE-1, the methylation levels in TSAs with HGD (55.7 ± 5.4) were significantly lower than those in TSAs without (58.8 ± 4.7) ($P = 0.024$).

Similarly, the methylation levels of *IGF2* DMR0 (52.0 ± 13.6) and LINE-1 (56.9 ± 5.5) in non-serrated adenomas with HGD were significantly lower than those in non-serrated adenomas without (59.0 ± 15.8, $P = 0.0016$ and 59.5 ± 5.9, $P = 0.0027$, respectively) (Table 3).

## DISCUSSION

In this study, we examined the *IGF2* DMR0 and LINE-1 methylation levels as well as other molecular alterations in 351 serrated lesions, 185 non-serrated adenomas, and 794 CRCs. *IGF2* DMR0 hypomethylation was less frequently detected in SSAs than in HPs, TSAs, and non-serrated adenomas. We also found that *IGF2* DMR0 and LINE-1 hypomethylation in TSAs and non-serrated adenomas with HGD were more frequently detected in TSAs and non-serrated adenomas without HGD, suggesting that hypomethylation may play an important role in the progression of these tumors.

In the current study, we confirmed that *IGF2* DMR0 hypomethylation was associated with poor CRC prognosis, suggesting its oncogenic role and malignant potential. In addition, our data showed that the *IGF2* DMR0 methylation level was inversely correlated with the IGF2 expression level. Therefore, our findings support the validity of the quantitative DNA methylation assay (bisulfite-pyrosequencing) for examining the *IGF2* DMR0 methylation level.

HPs are classified into three subtypes, namely microvesicular HPs, goblet cell HPs, and mucin-poor HPs. Microvesicular and goblet cell HPs are the most com-

**Table 3 Clinical and molecular features of sessile serrated adenomas with cytological dysplasia, traditional serrated adenomas, non-serrated adenomas (tubular or tubulovillous adenoma), and colorectal carcinomas according to disease stage *n* (%)**

| Clinical or molecular feature | Histological type | | | | | | | | | *P* value |
|---|---|---|---|---|---|---|---|---|---|---|
| | SSA with cytological dysplasia | Colorectal adenoma | | | | Colorectal carcinoma | | | | |
| | | TSA without HGD | TSA with HGD | Non-serrated adenoma without HGD | Non-serrated adenoma with HGD | Stage I | Stage II | Stage III | Stage IV | |
| All cases | 10 | 91 | 15 | 80 | 105 | 171 | 217 | 292 | 114 | |
| Sex | | | | | | | | | | |
| Male | 5 (50) | 55 (60) | 9 (60) | 53 (66) | 54 (51) | 107 (63) | 123 (57) | 168 (58) | 73 (64) | 0.50 |
| Female | 5 (50) | 36 (40) | 6 (40) | 27 (34) | 51 (49) | 64 (37) | 94 (43) | 124 (42) | 41 (36) | |
| Age (mean ± SD) | 74.1 ± 4.7 | 60.9 ± 12.3 | 62.7 ± 13.6 | 66.6 ± 11.2 | 66.3 ± 10.5 | 65.1 ± 11.0 | 67.4 ± 11.5 | 66.6 ± 12.5 | 63.4 ± 9.5 | 0.0016 |
| Tumor size (mm) (mean ± SD) | 12.3 ± 6.4 | 9.7 ± 4.7 | 12.8 ± 4.3 | 11.0 ± 7.4 | 29.3 ± 17.3 | 26.3 ± 15.8 | 53.1 ± 23.5 | 50.5 ± 22.7 | 50.9 ± 19.6 | < 0.0001 |
| Tumor location | | | | | | | | | | |
| Rectum | 0 (0) | 16 (18) | 5 (33) | 11 (14) | 23 (22) | 65 (38) | 73 (34) | 135 (46) | 37 (33) | < 0.0001 |
| Distal colon | 1 (10) | 39 (44) | 7 (47) | 26 (34) | 27 (26) | 44 (25) | 64 (29) | 59 (20) | 42 (37) | |
| Proximal colon | 9 (90) | 34 (38) | 3 (20) | 39 (51) | 54 (52) | 62 (36) | 80 (37) | 98 (34) | 34 (30) | |
| *BRAF* mutation | | | | | | | | | | |
| Wild-type | 2 (20) | 28 (31) | 7 (47) | 78 (98) | 102 (98) | 161 (95) | 204 (94) | 282 (97) | 103 (95) | < 0.0001 |
| Mutant | 8 (80) | 61 (69) | 8 (53) | 2 (2.5) | 2 (1.9) | 9 (5.3) | 13 (6.0) | 9 (3.0) | 6 (5.5) | |
| *KRAS* mutation | | | | | | | | | | |
| Wild-type | 10 (100) | 74 (83) | 11 (73) | 64 (80) | 48 (46) | 108 (64) | 145 (69) | 202 (70) | 84 (74) | < 0.0001 |
| Mutant | 0 (0) | 15 (17) | 4 (27) | 16 (20) | 57 (54) | 62 (36) | 66 (31) | 88 (30) | 29 (26) | |
| *PIK3CA* mutation | | | | | | | | | | |
| Wild-type | 10 (100) | 89 (100) | 14 (93) | 77 (99) | 99 (94) | 161 (94) | 194 (89) | 249 (85) | 103 (90) | < 0.0001 |
| Mutant | 0 (0) | 0 (0) | 1 (6.7) | 1 (1.3) | 6 (5.7) | 10 (5.9) | 23 (11) | 43 (15) | 11 (9.7) | |
| MSI status | | | | | | | | | | |
| MSS/MSI-low | 6 (60) | 90 (99) | 15 (100) | 80 (100) | 105 (100) | 163 (95) | 198 (91) | 276 (95) | 110 (96) | < 0.0001 |
| MSI-high | 4 (40) | 1 (1.1) | 0 (0) | 0 (0) | 0 (0) | 8 (4.7) | 19 (8.8) | 16 (5.5) | 4 (3.5) | |
| *IGF2* DMR0 methylation level (mean ± SD) | 69.0 ± 10.8 | 61.6 ± 19.6 | 50.2 ± 18.7 | 59.0 ± 15.8 | 52.0 ± 13.6 | 55.7 ± 15.8 | 53.4 ± 13.3 | 55.5 ± 12.9 | 53.1 ± 12.9 | < 0.0001 |
| LINE-1 methylation level (mean ± SD) | 58.3 ± 8.4 | 58.8 ± 4.7 | 55.7 ± 5.4 | 59.5 ± 5.9 | 56.9 ± 5.5 | 55.8 ± 7.2 | 53.1 ± 6.2 | 55.1 ± 6.5 | 54.1 ± 7.6 | < 0.0001 |

Percentage indicates the proportion of patients of each histological type who met the criteria for a specific clinical or molecular feature. *P* values were calculated by analysis of variance for age, tumor size, methylation levels of *IGF2* DMR0 and LINE-1 and by $\chi^2$ or Fisher's exact test for all other variables. The *P* value for significance was adjusted by Bonferroni correction to 0.0050 (= 0.05/10). HGD: High-grade dysplasia; HP: Hyperplastic polyp; MSI: Microsatellite instability; MSS: Microsatellite stable; SSA: Sessile serrated adenoma; TSA: Traditional serrated adenoma; *IGF2*: *Insulin-like growth factor* 2.

mon, whereas mucin-poor HPs are rare[44]. Recent studies have reported that microvesicular HPs may be a precursor lesion of SSAs and that borderline lesions between microvesicular HPs and SSAs can occur[25,26,28]. In the current study, we found that the *IGF2* DMR0 methylation levels of SSAs were significantly higher compared with those of HPs (microvesicular HPs), TSAs, and non-serrated adenomas. Our data also showed that *IGF2* DMR0 hypomethylation was less frequently detected in SSAs compared with HPs, TSAs, and non-serrated adenomas.

Our current study had some limitations due to its cross-sectional nature and the fact that unknown bias (*i.e.*, selection bias) may confound the results. Nevertheless, our multivariate regression analysis was adjusted for potential confounders including age, tumor size, tumor location, LINE-1 methylation level, and *BRAF* and *KRAS* mutation. The results demonstrate that *IGF2* DMR0 hypomethylation is inversely associated with SSAs. Moreover, our data have shown that the *IGF2* DMR0 methylation levels of SSAs with cytological dysplasia were higher than those of HPs, suggesting that HPs (microvesicular HPs) or SSAs with *IGF2* DMR0 hypomethylation may tend not to progress to the typical SSA pathway [HP-SSA-SSA with cytological dysplasia-carcinoma (MSI-high) sequence] but to the alternate pathway. Thus, our finding of differential patterns of *IGF2* DMR0 hypomethylation in serrated lesions may be a clue for elucidating the differentiation of serrated lesions.

In the current study, *IGF2* DMR0 hypomethylation was found in TSAs and hypomethylation was more frequently detected in TSAs with HGD when compared with TSAs without HGD. These results may imply that *IGF2* DMR0 hypomethylation can occur in the early stage of the TSA pathway and that TSAs with *IGF2* DMR0 hypomethylation are precursor lesions that progress to TSAs with HGD or CRCs with hypomethylation. In other words, TSAs without *IGF2* DMR0 hypomethylation may tend not to progress to TSAs with HGD. Otherwise, TSAs without *IGF2* DMR0 hypomethylation may tend to rapidly develop to CRCs; therefore, they are infrequently detected in the stage of TSA with HGD. However, because the number of TSA with HGD sam-

**Table 4 Clinicopathological and molecular features of fifteen traditional serrated adenomas with high-grade dysplasia**

| No. | Age/sex | Location | Size (mm) | *KRAS* mutation | *BRAF* mutation | *PIK3CA* mutation | *MGMT* methylation | *MLH1* methylation | MSI status | LINE-1 methylation level | *IGF2* DMR0 methylation level | IGF2 expression |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 75/M | Rectum | 8 | c.35G>A (p.G12D) | Wild | Wild | (-) | (-) | MSS/MSI-low | 58 | 70 | Weak |
| 2 | 54/F | Sigmoid colon | 20 | c.35G>A (p.G12D) | Wild | Wild | (+) | (-) | MSS/MSI-low | 53.7 | 39.5 | Strong |
| 3 | 62/F | Transverse colon | 15 | c.38G>A (p.G13D) | Wild | Wild | (+) | (-) | MSS/MSI-low | 53.3 | 72.0 | No expression |
| 4 | 84/M | Rectum | 5 | c.35G>A (p.G12D) | Wild | Wild | (-) | (-) | MSS/MSI-low | 65.0 | 26.5 | Moderate |
| 5 | 85/M | Sigmoid colon | 12 | Wild | c.1799T>A (p.V600E) | Wild | (-) | (-) | MSS/MSI-low | 58.0 | 45.5 | Strong |
| 6 | 48/M | Sigmoid colon | 20 | Wild | c.1799T>A (p.V600E) | Wild | (-) | (-) | MSS/MSI-low | 53.7 | 40.5 | Moderate |
| 7 | 69/M | Sigmoid colon | 10 | Wild | c.1799T>A (p.V600E) | Wild | (-) | (-) | MSS/MSI-low | 58.7 | 52.0 | No expression |
| 8 | 60/M | Descending colon | 9 | Wild | c.1799T>A (p.V600E) | Wild | (-) | (-) | MSS/MSI-low | 59.0 | 41.5 | Moderate |
| 9 | 34/M | Sigmoid colon | 18 | Wild | c.1799T>A (p.V600E) | Wild | (+) | (-) | MSS/MSI-low | 57.3 | 42.0 | Strong |
| 10 | 61/M | Rectum | 10 | Wild | c.1799T>A (p.V600E) | Wild | (-) | (-) | MSS/MSI-low | 56.7 | 29.0 | Strong |
| 11 | 52/F | Ascending colon | 15 | Wild | c.1799T>A (p.V600E) | Wild | (+) | (+) | MSS/MSI-low | 57.0 | 57.0 | Moderate |
| 12 | 70/F | Rectum | 13 | Wild | c.1799T>A (p.V600E) | Wild | (+) | (+) | MSS/MSI-low | 63.0 | 84.5 | Weak |
| 13 | 66/F | Ascending colon | 12 | Wild | Wild | c.1624G>A (p.E542K) | (-) | (-) | MSS/MSI-low | 49.7 | 28.0 | Moderate |
| 14 | 52/M | Sigmoid colon | 12 | Wild | Wild | Wild | (-) | (-) | MSS/MSI-low | 48.0 | 44.5 | Moderate |
| 15 | 69/F | Rectum | 13 | Wild | Wild | Wild | (+) | (-) | MSS/MSI-low | 44.3 | 80.0 | Weak |

HGD: High-grade dysplasia; MSI: Microsatellite instability; MSS: Microsatellite stable; TSA: Traditional serrated adenoma.

ples was small ($n$ = 15), our findings require further confirmation from future independent studies.

Global DNA hypomethylation is associated with genomic instability, which leads to cancer[45-50]. As the LINE-1 or L1 retrotransposon constitutes a substantial portion (ca. 17%) of the human genome, the level of LINE-1 methylation is regarded as a surrogate marker of global DNA methylation[46,51]. We previously reported that LINE-1 methylation is highly variable but is strongly associated with a poor prognosis in CRC[45]. However, no previous study has reported the role of LINE-1 hypomethylation in serrated lesions. In serrated lesions, unlike the *IGF2* DMR0 methylation level, no significant difference was observed between the LINE-1 methylation level and histological type. We also found that the LINE-1 methylation levels in TSAs with HGD were significantly lower than those in TSAs. These results suggest that both *IGF2* DMR0 hypomethylation and LINE-1 hypomethylation are important epigenetic alterations in the progression of TSAs. Because the carcinogenic mechanism remains unclear, further analyses are needed to clarify the role in the TSA pathway of the hypomethylation of these locations.

Previous studies have reported that SSAs with cytological dysplasia have accumulated genetic abnormalities and are at a high risk of progression to colorectal carcinoma[7,26,28]. Loss of staining for MLH1 leads to MSI and repeat tract mutation in genes such as *TGFβRII* is restricted to lesions with cytological dysplasia in SSAs[26,27,52,53]. In the current study, MSI-high was more frequently detected in SSAs with cytological dysplasia than in SSAs without. Our data indicate that in SSAs with cytological dysplasia, it is not hypomethylation of *IGF2* DMR0 or LINE-1 but rather MSI due to *MLH1* hypermethylation that plays an important role in the evolution to colorectal carcinoma.

The RAS-RAF-MEK-ERK signaling pathway is commonly altered in CRC and serrated lesions through oncogenic mutation of either *BRAF* or *KRAS*[15,21,25]. Moreover, CRCs with serrated morphology are particularly prone to mutations targeted by anti-epidermal growth factor receptor therapy. Therefore, as the variety of molecularly targeted agents for CRC increases, understanding of molecular alterations is becoming increasingly important[21,40]. *BRAF* and *KRAS* mutations are mutually exclusive and demonstrate a subtype specificity in serrated lesions[10,15,17-19,28]; they are most likely initiating events in the majority of HPs[54]. Previous studies have reported that *BRAF* is mutated with increasing frequency in SSAs (60%-100%)[3-5,9,11,16]. In the current study, *BRAF* mutations were detected in 49% of HPs and 87% of SSAs, respectively. Therefore, our data relating to the frequency of *BRAF* mutations in SSAs are consistent with previous reports. The activation of the RAS-RAF-MEK-ERK signaling pathway by *BRAF* or *KRAS* mutation is also common in TSAs. Previous studies have reported *BRAF* mutation rates in TSAs ranging from 27% to 55%[6,8,16,55], compared to *KRAS* mutation rates of 29%-46%[6,8]. In the cur-

rent study, *BRAF* and *KRAS* mutations were detected in 69% and 17% of TSAs, respectively. Thus, the wide variation in the relative proportion of *BRAF vs KRAS* mutations in different studies reflects differences in histological classification or small sample size.

In conclusion, we found that *IGF2* DMR0 hypomethylation can occur in the early stage of any histological types of serrated lesions; however, hypomethylation may be an infrequent epigenetic alteration in SSAs. These results imply that *IGF2* DMR0 hypomethylation may be a key epigenetic event that affects the progression of HPs. Our data also suggest that the hypomethylation of *IGF2* DMR0 and LINE-1 may play an important role in the progression of the TSA pathway.

## COMMENTS

### Background

The serrated pathway attracts considerable attention as an alternative colorectal cancer (CRC) pathway. Authors previously reported the association of *insulin-like growth factor 2* (*IGF2*) differentially methylated region (DMR)0 hypomethylation with poor prognosis and its link to global DNA hypomethylation [long interspersed nucleotide element-1 (LINE-1) hypomethylation] in CRC; however, to date, there have been no studies describing its role in the serrated pathway.

### Research frontiers

Sessile serrated adenoma (SSA) and traditional serrated adenoma (TSA) are premalignant lesions, but SSA is the principal serrated precursor of CRC. In particular, there are many clinicopathological and molecular similarities between SSA and microsatellite instability (MSI)-high CRC, for example, right-sided predilection, *MLH1* hypermethylation, and frequent *BRAF* mutation. Therefore, SSAs are hypothesized to develop in some cases to MSI-high CRCs with *BRAF* mutation in the proximal colon. In contrast, a definite precursor of TSA has not been established. In addition, the key carcinogenic mechanism involved in this TSA pathway remains largely unknown. To investigate the role of *IGF2* DMR0 hypomethylation in serrated lesions they examined *IGF2* DMR0 methylation levels as well as other molecular alterations.

### Innovations and breakthroughs

This is the first report of an association between histopathological findings and *IGF2* DMR0 hypomethylation in serrated lesions. *IGF2* DMR0 hypomethylation was less frequently detected in SSAs than in hyperplastic polyps (HPs), TSAs, and non-serrated adenomas. They also found that *IGF2* DMR0 and LINE-1 hypomethylations in TSAs and non-serrated adenomas with high-grade dysplasia were more frequently detected in TSAs and non-serrated adenomas, suggesting that such hypomethylation may play an important role in the progression of those tumors. Thus, their finding of differential patterns of *IGF2* DMR0 hypomethylation in serrated lesions may be a clue for elucidating the progression of serrated lesions.

### Applications

In the current study, authors found that the *IGF2* DMR0 methylation levels of SSAs were significantly higher compared with those of HPs (microvesicular HPs), TSAs, and non-serrated adenomas. They also showed that *IGF2* DMR0 hypomethylation was less frequently detected in SSAs compared with HPs, TSAs, and non-serrated adenomas. Therefore, their data challenge the common conception of discrete molecular features of SSAs *vs* other serrated lesions (TSAs and HPs) and may have a substantial impact on clinical and translational research, which has typically been performed with the dichotomous classification of SSAs.

### Terminology

*IGF2* DMR: IGF2 expression is controlled by CpG-rich regions known as *IGF2* DMRs in CRC. In particular, *IGF2* DMR0 hypomethylation has been suggested as a surrogate-biomarker for *IGF2* loss of imprinting. LINE-1: Global DNA hypomethylation is associated with genomic instability, which leads to cancer. As the long interspersed nucleotide element-1 or L1 retrotransposon constitutes a substantial portion of the human genome, the level of LINE-1 methylation is regarded as a surrogate marker of global DNA methylation. Serrated pathway: The serrated neoplasia pathway has attracted considerable attention as an alternative pathway of CRC development, and serrated lesions exhibit unique clinicopathological or molecular features. Of the serrated lesions, SSAs are hypothesized to develop in some cases to MSI-high CRCs with *BRAF* mutation in the proximal colon.

### Peer review

The authors investigated the hypomethylations of *IGF2* DMR0 and LINE-1; MSI; and mutations of *KRAS*, *BRAF*, and *PIK3CA* in patients with serrated lesions and non-serrated adenomas. The results demonstrated that *IGF2* DMR0 hypomethylation can occur in the early stage of any histological types of serrated lesions; however, hypomethylation may be an infrequent epigenetic alteration in SSAs. The authors also revealed that the hypomethylation of *IGF2* DMR0 and LINE-1 may play an important role in the progression of the TSA pathway. This article may have a substantial impact on clinical and translational research in the progression of serrated lesions related to malignant transformation.

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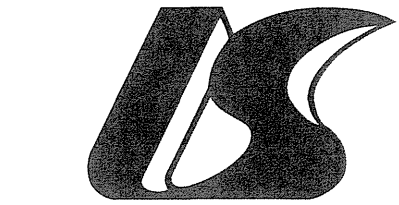

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RAPID COMMUNICATION

# CCAAT/enhancer binding protein α (C/EBPα)$^+$ M2 macrophages contribute to fibrosis in IgG4-related disease?

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**Abstract**

IgG4-related disease (IgG4-RD) is a new disease entity characterized by type 2 helper T (Th2)-dominant inflammation and progressive fibrosis. We found the infiltration of strange cell populations in the fibrotic lesions of submandibular gland specimens obtained from 15 patients with IgG4-RD. These cells expressed CCAAT/enhancer binding protein a (C/EBPα). Many of the cell populations were identified with M2 macrophages. The degrees of infiltration of C/EBPα$^+$M2 macrophages and the ratio of fibrotic lesions in the specimens were correlated ($r^2 = 0.83$, $p < 0.01$). We also analyzed the expression of C/EBPα in other chronic inflammatory disorders: synovium in rheumatoid arthritis (RA), liver tissue in chronic viral hepatitis, and mucosa in ulcerative colitis. The specimens from RA and chronic viral hepatitis showed infiltration of C/EBPα$^+$ cells, but there were few C/EBPα-positive cells in ulcerative colitis. Fibrosis is not a major issue in ulcerative colitis. In conclusion, we found the remarkable infiltration of C/EBPα$^+$M2 macrophages in cases of chronic inflammation with fibrosis, including IgG4-RD. This primitive study also disclosed that most of C/EBPα$^+$M2 macrophages localized in fibrotic lesions, and the degree of the infiltration and the ratio of fibrotic area were correlated.

**Keywords**

CCAAT/enhancer binding protein α, Fibrosis, IgG4-related disease, Macrophage

**History**

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Organ fibrosis occurs and proceeds irreversibly with many chronic inflammatory disorders. It often becomes clinically problematic in pulmonary fibrosis, liver cirrhosis, and diabetic nephropathy. Fibrosis also occurs in inflammatory bowel diseases and rheumatic disorders. Extracellular matrix is deposited by activated fibroblasts in the fibrosis, but the mechanisms of the fibrosis remain the critical issue to be elucidated.

IgG4-related disease (IgG4-RD) is a new disease entity characterized by elevated levels of serum IgG4 and remarkable infiltration of IgG4-bearing plasmacytes and fibrosis in the involved organs. We have investigated the peculiar pathogenesis of type 2 helper T (Th2)-dominant inflammation and progressive fibrosis [1]. During our research, we found the infiltration of strange cell populations in the fibrotic lesions of submandibular gland specimens obtained from 15 patients with IgG4-RD. Clinical and histopathological data was shown in Table 1. These cells expressed CCAAT/enhancer binding protein α (C/EBPα) (anti-CEBPA antibody, Fitzgerald Industries International, Inc. North Acton, MA, USA). Many of the cell populations were identified with M2 macrophages as they expressed CD163 (anti-CD163 antibody, Epitomics. Burlingame, CA, USA) (Figure 1a, b), but all M2 macrophages did not express C/EBPα. Microscopic images of all cases were digitalized (DP Controller version 2.3.1.231; Olympus, Tokyo, Japan) and then Scion Image version 4.0.3.2 software (Scion Corporation, Frederick, MD, USA) was used to assess the fibrotic lesions. The degrees of infiltration of C/EBPα$^+$M2 macrophages and the ratio of fibrotic lesions in the specimens were correlated ($r^2 = 0.83$, $p < 0.01$). We also analyzed the expression of C/EBPα in other chronic inflammatory disorders: synovium in rheumatoid arthritis (RA), liver tissue in chronic viral hepatitis, and mucosa in ulcerative colitis. We examined five cases for each disease. C/EBPα-positive cells were observed in synovium of RA (30–50/HPF) and in the liver tissue of chronic hepatitis (20–40/HPF), which presented with chronic inflammation with fibrosis. On the other hand, there were few C/EBPα-positive cells in ulcerative colitis. Fibrosis is not a major issue in ulcerative colitis (Figure 2a–c).

C/EBPα is expressed on the terminally differentiated cells of various tissues, including the hematopoietic system, liver, adipocytes, and lungs, and plays an important role in cellular differentiation. C/EBPα is expressed on myeloid progenitor cells in the hematopoietic system. Blood cell differentiation tends to be inversely correlated with the degree of C/EBPα expression. C/EBPα is a crucial transcription factor

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Table 1. Clinical and pathological findings of 15 cases with IgG4-related disease.

| Case No. | Age | Sex | Analyzed organ | Serum IgG4 (mg/dL) | CD163 (cells/HPF) | C/EBPα (cells/HPF) | CD163+ C/EBP α + (cells/HPF) | Distribution of CD163+C/EBPα + | Ratio of fibrosis in specimen (%) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 69 | M | SM | 173 | 78 | 42 | 26 | IF | 53.4 |
| 2 | 67 | M | SM | 626 | 26 | 28 | 12 | IF | 27.2 |
| 3 | 64 | M | SM | 1410 | 58 | 20 | 9 | IF | 19.8 |
| 4 | 57 | M | SM | 402 | 55 | 12 | 7 | IF | 17.5 |
| 5 | 64 | M | SM | 257 | 28 | 8 | 7 | IF | 14.6 |
| 6 | 51 | F | SM | 768 | 62 | 23 | 5 | IF | 9.5 |
| 7 | 65 | M | SM | 548 | 46 | 36 | 13 | IF | 16.7 |
| 8 | 74 | M | SM | 374 | 38 | 16 | 14 | IF | 17.9 |
| 9 | 64 | M | SM | 2210 | 61 | 12 | 11 | IF | 20.4 |
| 10 | 69 | M | SM | 1420 | 36 | 13 | 13 | IF | 15.6 |
| 11 | 55 | F | SM | 870 | 39 | 8 | 6 | IF | 8.9 |
| 12 | 74 | F | SM | 963 | 21 | 3 | 3 | IF | 6.0 |
| 13 | 62 | M | SM | 796 | 26 | 11 | 10 | IF | 18.2 |
| 14 | 71 | M | SM | 457 | 39 | 24 | 18 | IF | 25.4 |
| 15 | 57 | M | SM | 329 | 33 | 26 | 18 | IF | 36.2 |

*HPF* high power field, *C/EBPα* CCAAT/enhancer binding protein α, M male, F female, SM submandibular gland, IF interfollicular.



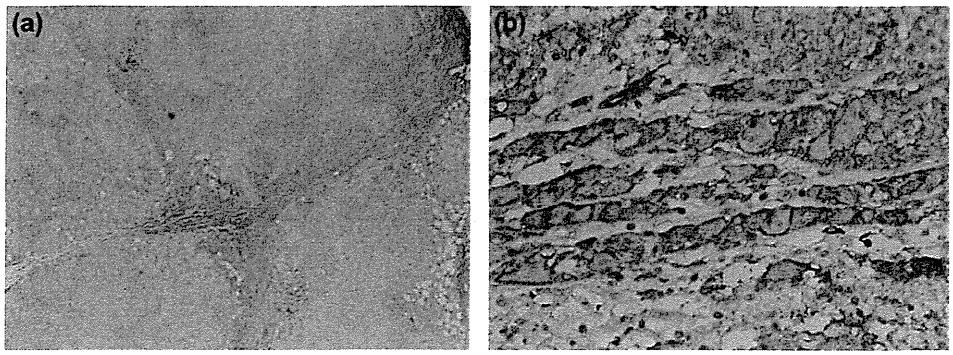


Figure 1. Identification of cells expressing C/EBPα. Double immunostaining with CD163 (*red*) and CCAAT/enhancer binding protein α (C/EBPα) (*brown*) in the submandibular glands of IgG4-related disease. C/EBPα-positive cells are observed along the fibrosis. These cells express CD163 (**a.** magnification ×40, **b.** magnification ×400).

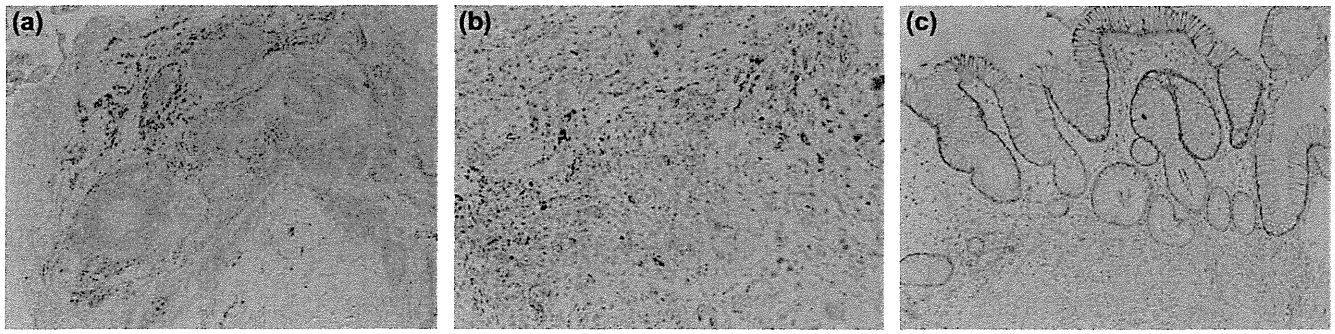


Figure 2. Distribution of C/EBPα-positive cells in other diseases. (a) Rheumatoid arthritis (magnification x40). (b) Liver cirrhosis due to hepatitis B virus (magnification ×200). (c) Ulcerative colitis (magnification x100). C/EBPα-positive cells are observed in the synovium of rheumatoid arthritis and cirrhotic liver tissues presenting fibrosis. In ulcerative colitis, for which fibrosis is not really a problem, there is little C/EBPα expression.

in the differentiation from common myeloid progenitor (CMP) to granulocyte/macrophage progenitor (GMP) and is no longer expressed in mature granulocytes and macrophages. It is not usually important in lymphoid differentiation [2].

The origin and the role of C/EBPα$^+$M2 macrophages are unknown. It was recently reported that C/EBPα could mediate the transdifferentiation of pre-B cells to macrophages [3,4]. If this transdifferentiation occurs in chronic inflammatory disorders with fibrosis, the origin of C/EBPα$^+$M2 macrophages could be pre-B cells in the bone marrow. Rituximab is an effective treatment for IgG4-RD [5]; but, the target of anti-CD20 antibodies in IgG4-RD is unclear because IgG4-RD is characterized by Th2-dominant inflammation and fibrosis. Rituximab can be transferred to the bone marrow. If our hypothesis is correct, one of the mechanisms by which rituximab regulates the pathogenesis of IgG4-RD is by depletion of pre-B cells in the bone marrow, which would prevent the transdifferentiation of pre-B cells into macrophages by C/EBPα. We must prove this hypothesis with experimental animals and further analyze other diseases characterized by fibrosis in our next studies.

With respect to the role of C/EBPα$^+$M2 macrophages, it is known that macrophages contribute to fibrosis [6]. This primitive study disclosed that most of C/EBPα$^+$M2 macrophages localized in fibrotic lesions, and the degree of the infiltration and the ratio of fibrotic area were correlated. Macrophages are classified as M1 macrophages, which contribute to

inflammation, and M2 macrophages, which play a role in tissue repair. M2 macrophages are mainly involved in the fibrosis. It is also unknown that C/EBPα$^+$M2 macrophages are involved in RA. There are many synovial fibroblasts, but few fibrosis in the synovium of RA. Over production of matrix metalloproteinase (MMP) leads to collagen degradation in RA [7]. It is considered that cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and transforming growth factor (TGF)-β regulate the expression of mRNA of MMP [8]. It is estimated that in those cytokines environments fibrosis is difficult to occur. C/EBPα$^+$ cells were not detected in the specimens with ulcerative colitis. It was considered that the factors other than the polarity of Th1-Th2 are also present. Research about the relationship between macrophages and fibrosis in cirrhosis is in progress. The inhibition of macrophage migration inhibitory factor (MIF) can lead to suppression of liver fibrosis [9]. It is important to analyze how fibrosis is affected by the presence or absence of C/EBPα expression.

In conclusion, we found the remarkable infiltration of C/EBPα$^+$M2 macrophages in cases of chronic inflammation with fibrosis, including IgG4-RD. This is the first report of C/EBPα$^+$M2 macrophages being present in fibrosis. It is necessary to examine the function and regulation of this cell group in fibrosis.

## Conflict of interest

None.

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1. Introduction
2. DNA synthesis inhibitors
3. Antimetabolites
4. Platinum analogs
5. Conclusion
6. Expert opinion





# DNA synthesis inhibitors for the treatment of gastrointestinal cancer

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***Introduction:*** Intensive laboratory, preclinical and clinical studies have identified and validated molecular targets in cancers, leading to a shift toward the development of novel, rationally designed and specific therapeutic agents. However, gastrointestinal cancers continue to have a poor prognosis, largely due to drug resistance.

***Areas covered:*** Here, we discuss the current understanding of DNA synthesis inhibitors and their mechanisms of action for the treatment of gastrointestinal malignancies.

***Expert opinion:*** Conventional agents, including DNA synthesis inhibitors such as fluoropyrimidines and platinum analogs, remain the most effective therapeutics and are the standards against which new drugs are compared. Novel DNA synthesis inhibitors for the treatment of gastrointestinal malignancies include a combination of the antimetabolite TAS-102, which consists of trifluorothymidine with a thymidine phosphorylase inhibitor, and a novel micellar formulation of cisplatin NC-6004 that uses a nanotechnology-based drug delivery system. The challenges of translational cancer research using DNA synthesis inhibitors include the identification of drugs that are specific to tumor cells to reduce toxicity and increase antitumor efficacy, biomarkers to predict pharmacological responses to chemotherapeutic drugs, identification of ways to overcome drug resistance and development of novel combination therapies with DNA synthesis inhibitors and other cancer therapies, such as targeted molecular therapeutics. Here, we discuss the current understanding of DNA synthesis inhibitors and their mechanisms of action for the treatment of gastrointestinal malignancies.

**Keywords:** antimetabolite, DNA synthesis inhibitor, drug delivery system, drug resistance, platinum analogs, translational cancer research

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## 1. Introduction

Cancer is a major public health problem in the US and other developed countries. DeSantis *et al.* reported that 1,665,540 new cancer cases are expected in the US in 2014 [1].Gastrointestinal cancer refers to malignancy of the gastrointestinal tract and accessory organs involved in digestion, including the esophagus, stomach, biliary system, pancreas, small intestine, colon, rectum and anus. An estimated 18,170 new cases of esophageal cancer, 22,220 new cases of stomach cancer, 136,830 new cases of colon and rectal cancer, 46,420 new cases of pancreatic cancer, 9,160 new cases of small intestine cancer and 33,190 new cases of liver and intrahepatic bile duct cancer will be diagnosed in 2014. Despite advances in surgery, radiation therapy, systemic chemotherapy and supportive therapies, the 5-year relative survival rates for all cancer in the US is ~ 66% for patients diagnosed between

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**Article highlights.**

- TAS-102 is a novel combination antimetabolite which consists of trifluorothymidine with a thymidine phosphorylase inhibitor.
- NC-6004 is a novel micellar formulation of cisplatin which uses a nanotechnology-based drug delivery system.
- The challenges of translational cancer research using DNA synthesis inhibitors include the identification of drugs that are specific to tumor cells, biomarkers to predict pharmacologic responses, identification of ways to overcome drug resistance, and development of novel combination therapies.

This box summarizes key points contained in the article.



2003 and 2009, and followed through 2010. Thus, the development of novel cancer therapeutics is urgently needed to improve cancer prognosis.

According to the American Cancer Society, cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells. It is caused by the accumulation of genetic mutations and epigenetic alterations in oncogenes and tumor suppressor genes [2,3]. Cancer chemotherapy has changed since curative treatments were identified for previously fatal malignancies with rapid cell growth, such as acute leukemia [4]. As many chemotherapies affect mitosis, tumors with high growth rates are more sensitive to chemotherapy because a larger proportion of the targeted cells are undergoing cell division at any time. However, conventional chemotherapy is less effective against slow growing cancers, including gastrointestinal cancers. Additionally, intratumoral heterogeneity may contribute to the varying sensitivity of cancer cells to chemotherapy, as well as to drug resistance [5].

There are a number of strategies in the administration of chemotherapeutic drugs, including combination chemotherapy, combined modality chemotherapy, postoperative (adjuvant) chemotherapy, preoperative (neoadjuvant) chemotherapy and salvage chemotherapy. Chemotherapy is also employed as part of the multimodal treatment of cancer, such as esophageal cancer, thereby allowing for more limited surgery. Adjuvant and neoadjuvant chemotherapy can extend life and prevent disease recurrence following surgical resection of gastrointestinal cancers, including esophageal, gastric, colorectal and pancreatic cancer [6].

Recently, there has been a shift toward developing novel, rationally designed and specific agents for cancer therapy [2,7,8] Among gastrointestinal cancers, there are novel molecularly targeted therapeutics, including the tyrosine kinase inhibitors imatinib and sunitinib for gastrointestinal stromal tumors [9,10], regorafenib for metastatic colorectal cancer [11] and gastrointestinal stromal tumors [12], sunitinib and everolimus for pancreatic neuroendocrine tumors and erlotinib in combination with gemcitabine for advanced pancreatic carcinoma [13]. Additionally, therapeutic monoclonal antibodies have been developed, including a humanized anti-VEGF monoclonal antibody, bevacizumab, for metastatic colorectal cancer [14], a chimeric anti-EGFR monoclonal antibody, cetuximab, for metastatic colorectal cancer [15], a human monoclonal antibody to EGFR, panitumumab, for metastatic colorectal cancer, a humanized anti-Her2 receptor monoclonal antibody, trastuzumab, for metastatic gastric or gastroesophageal junction adenocarcinoma [16,17] and a human monoclonal antibody to the Her2 receptor, ramucirumab, for metastatic gastric or gastroesophageal junction adenocarcinoma. Moreover, recombinant fusion proteins have been developed, such as ziv-aflibercept, consisting of the binding portions of VEGF from VEGF receptors 1 and 2 fused to the Fc portion of immunoglobulin G1, for metastatic colorectal cancer [18]. However, despite the remarkable successes of the molecularly targeted agents discussed above, the prognosis of gastrointestinal cancer remains poor due to drug resistance.

New therapies for gastrointestinal cancers are not likely to replace cytotoxic agents, many of which act by damaging DNA. Rather, cytotoxic agents combined with molecularly targeted drugs will continue to be used in chemotherapy for gastrointestinal cancers. Here, we discuss the current understanding of DNA synthesis inhibitors and their mechanisms of action for the treatment of gastrointestinal cancers in order to improve patient prognosis.

## 2. DNA synthesis inhibitors

Traditionally, cancer drugs have been discovered through large-scale testing of synthetic chemicals and natural products in proliferating animal tumor systems, including mouse allograft preclinical cancer models using murine leukemia cells, human xenograft models using immunodeficient mice and *in vitro* human cancer cell line models, such as the anticancer drug screen conducted in 60 human tumor cell lines by the United States National Cancer Institute (NCI) [4,19]. Over time, this system has evolved into one that combines both *in vitro* human cancer cell lines with human xenograft models. Most of the agents discovered in these drug screens interact with DNA or its precursors, inhibiting the synthesis of new genetic material and causing damage to DNA in both normal and malignant cells. Unfortunately, none of the screening systems have successfully predicted outcome of clinical trials [20,21].

The drugs used in cancer chemotherapy are varied in structure and mechanism of action. Most chemotherapeutic drugs work by impairing mitosis, effectively targeting fast-dividing cells. These drugs prevent mitosis through a number of mechanisms, including damaging DNA and inhibiting the cellular machinery involved in cell division. Interestingly, many of these drugs inhibit DNA synthesis.

DNA synthesis is the creation of new DNA molecules through the process of DNA replication, wherein a replication initiator protein splits the existing cellular DNA and makes a copy of each split strand. The copied strands are then joined together with their template strand to form a new DNA molecule. DNA replication proceeds in three enzymatically

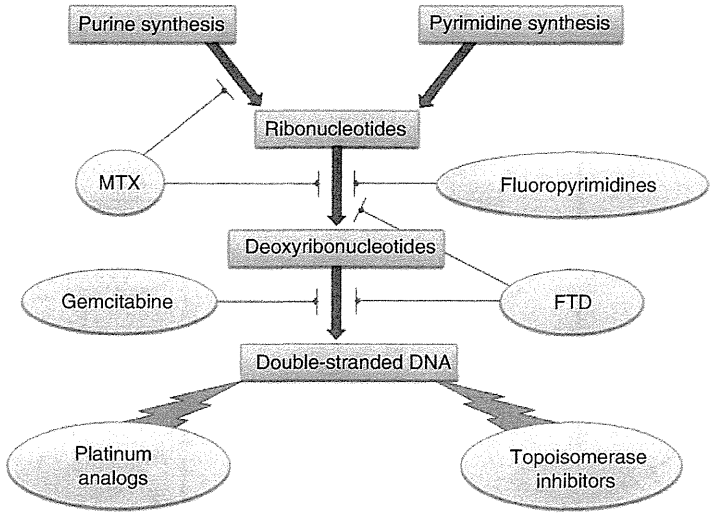


**Figure 1. The mechanisms and sites of action of DNA synthesis inhibitors.** The folic acid analog MTX inhibits dihydrofolate reduction, blocks thymidylate and purine synthesis, and interrupts the synthesis of DNA and RNA. Pyrimidine analogs fluoropyrimidines, such as 5-fluorouracil, inhibit thymidylate synthesis. The pyrimidine analog gemcitabine incorporates into DNA, thereby interfering with DNA synthesis. The pyrimidine analog FTD, part of the novel oral formulation TAS-102, incorporates into DNA as well as inhibits thymidine synthesis. Platinum analogs, such as cisplatin, form adducts with DNA. Topoisomerase inhibitors, such as camptothecin and epirubicin, block topoisomerase function.
FTD: α,α,α-Trifluorothymidine; MTX: Methotrexate.



catalyzed and coordinated steps: initiation, elongation and termination.

DNA synthesis inhibitors include antimetabolite analogs of folic acid, pyrimidine and purine. Figure 1 summarizes the mechanisms and sites of action of DNA synthesis inhibitors [4,22].The folic acid analog methotrexate (MTX) inhibits dihydrofolate reduction, blocks thymidylate and purine synthesis, and interrupts the synthesis of DNA and RNA. Fluoropyrimidines, a group of pyrimidine analogs that include 5-fluorouracil (5-FU), inhibit thymidylate synthesis. The pyrimidine analog gemcitabine incorporates into DNA, thereby interfering with DNA synthesis. The pyrimidine analog α, α,α-trifluorothymidine (FTD or TFT), a part of the novel oral formulation TAS-102, incorporates into DNA and inhibits thymidine synthesis [23]. Platinum analogs, such as cisplatin, form covalent adducts between platinum-DNA, which inhibit fundamental cellular processes, including DNA replication, transcription, translation and DNA repair [24].Topoisomerase inhibitors such as camptothecin and epirubicin interfere with the action of topoisomerase enzymes, which regulate the overwinding or underwinding of DNA. In this review, we discuss the antimetabolite and platinum analog DNA synthesis inhibitors in gastrointestinal cancers.

## 3. Antimetabolites

Antimetabolites were among the first effective chemotherapeutic agents discovered [22]. Their structures are similar to the molecules used in nucleic acid synthesis. As a result, they inhibit the enzymes needed for nucleic acid synthesis and/or are incorporated into DNA and RNA macromolecules to induce cell death during S phase, the part of the cell cycle in which DNA is replicated. Because cancer cells divide more rapidly than normal cells, antimetabolites affect the replication of cancer cells to a greater extent than normal cells. Pyrimidine analogs, purine analogs and folate antagonists are the main categories of antimetabolites.

### 3.1 Pyrimidine analogs

Fluoropyrimidines were developed in the 1950s following the observation that rat hepatomas used the pyrimidine uracil more rapidly than normal tissues, indicating that uracil metabolism was a potential target for antimetabolite chemotherapy [25].

5-FU is an analog of uracil with a fluorine atom at the C-5 position in place of hydrogen. 5-FU rapidly enters the cell using the same facilitated transport mechanism as uracil. Since its development by Heidelberger *et al.* in 1957, it has been used as a standard chemotherapy for solid tumors, such as gastrointestinal cancers [26]. The mechanism of 5-FU cytotoxicity has been ascribed to the misincorporation of its metabolites into RNA and DNA, and to the inhibition of the nucleotide synthesizing enzyme thymidylate synthase (TS).

5-FU is converted to three active metabolites (Figure 2): fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP) and fluorouridine triphosphate

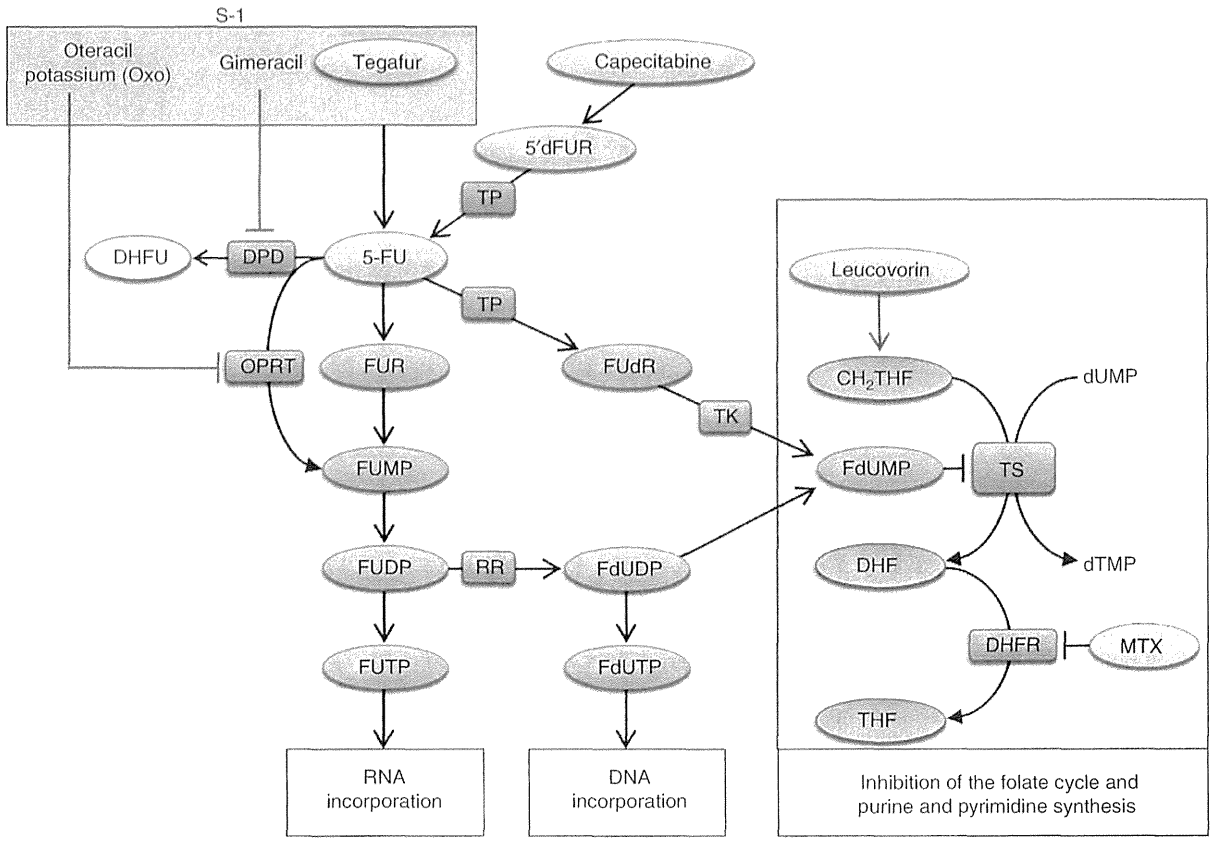


**Figure 2. Summary of the metabolism of fluoropyrimidines. 5-FU is converted to three active metabolites: FdUMP, FdUTP, and FUTP.** These active metabolites disrupt the synthesis of DNA and RNA and the action of TS. The main mechanism of 5-FU activation is conversion to FdUMP to inhibit TS, which plays an important role in the folate cycle and purine and pyrimidine synthesis. Addition of exogenous folate in the form of folinic acid (leucovorin) increases the intracellular pool of $CH_2THF$, thereby enhancing TS inhibition by FdUMP. The conversion of 5-FU to FdUMP can occur either directly via TP to FUdR and then to FdUMP by TK, or indirectly via FUR or FUMP to FUDP, and then to FdUDP and FdUMP by RR. FUDP and FdUDP can also be converted to FUTP and FdUTP to incorporate into RNA and DNA, respectively, which contributes to the cytotoxicity of 5-FU. DPD mediates the conversion of 5-FU to DHFU. Gimeracil inhibits DPD-mediated degradation of 5-FU. Capecitabine is a 5-FU pro-drug that is converted to 5'dFUR, which is then converted to 5-FU by TP. S-1 combines the 5-FU prodrug tegafur, a DPD inhibitor gimeracil, and an orotate phosphoribosyltransferase inhibitor oteracil potassium to improve the selectivity of action of 5-FU. MTX inhibits DHFR, inhibit dihydrofolate reduction to THF, and block thymidylate and purine synthesis.

5-FU: 5-Fluorouracil; $CH_2THF$: 5,10-Methylene tetrahydrofolate; 5'dFUR; 5'-Deoxy-5-fluorouridine; DHF: Dihydrofolate; DHFR: Dihydrofolate reductase; DHFU: Dihydrofluorouracil; DPD: Dihydropyrimidine dehydrogenase; FdUMP: Fluorodeoxyuridine monophosphate; FdUTP: Fluorodeoxyuridine triphosphate; FUDP: Fluorouridine diphosphate; FUdR; Fluorodeoxyuridine; FUMP; Fluorouridine monophosphate; FUR: Fluorouridine; FUTP: Fluorouridine triphosphate; MTX: Methotrexate; RR: Ribonucleotide reductase; THF: Tetrahydrofolates; TK: Thymidine kinase; TP: Thymidylate phosphorylase; TS: Thymidylate synthase.

(FUTP). The main mechanism of 5-FU activation is via conversion to FdUMP, leading to TS inhibition and inhibition of the folate cycle and purine and pyrimidine synthesis. Inhibition of TS by FdUMP in the presence of 5,10-methylene tetrahydrofolate ($CH_2THF$) results in the depletion of thymidine triphosphate and the elevation of deoxyadenosine-5'-triphosphate (dATP), which induces DNA damage, S-phase arrest and apoptosis. The addition of exogenous folate in the form of folinic acid (leucovorin) increases the intracellular pool of $CH_2THF$, thereby enhancing FdUMP-induced TS inhibition. Thus, 5-FU with leucovorin is a standard combination to enhance the antineoplastic activity of 5-FU [27].

The conversion of 5-FU to FdUMP can occur directly via thymidylate phosphorylase (TP)-mediated conversion to fluorodeoxyuridine, followed by thymidine kinase-mediated conversion to FdUMP. FdUMP conversion can also occur indirectly through the conversion of fluorouridine or fluorouridine monophosphate to fluorouridine diphosphate (FUDP), and then ribonucleotide reductase (RR)-mediated conversion to FdUDP and FdUMP. FUDP and FdUDP

can also be converted to FUTP and FdUTP and incorporated into RNA and DNA, respectively, which can contribute to cytotoxicity by fluoropyrimidines. Incorporation of 5-FUTP into RNA interferes with RNA processing and is considered to be the primary mechanism of gastrointestinal toxicity. It is also a dose-limiting toxicity during continuous venous administration of 5-FU. Nevertheless, incorporation of FdUTP into DNA induces cytotoxicity, which is important in the chemotherapeutic response [28].

Dihydropyrimidine dehydrogenase (DPD)-mediated conversion of 5-FU to dihydrofluorouracil is the rate-limiting step in 5-FU catabolism. Because of this catabolism, 85% of dosed 5-FU is metabolized to α-fluoro-β-alanine, with an elimination half-life of 10 – 20 min, thus preventing its antitumor effect [29].Importantly, DPD inhibitors, such as gimeracil, inhibit DPD-mediated degradation of 5-FU, enhancing its antitumor activity. Because of its enhanced antitumor activity, DPD inhibitors have been added to combination therapies such as S-1, discussed below [30]. Importantly, continuous intravenous infusion of 5-FU for 24 – 120 h achieves steady plasma concentration and has more effective antitumor activity than intravenous bolus administration [31].

Oral administration of chemotherapeutic drugs can achieve steady plasma concentration and is beneficial in that it enables patients to receive treatment as outpatients and to maintain their quality of life. An oral formulation of fluorouracil was developed in the 1970s [32].Tegafur or ftorafur (1-(2-tetrahydrofuryl)-5-FU), an oral prodrug metabolized in the liver to 5-FU by cytochrome P450 2A6, was developed by Giller *et al.* [33,34]. In order to optimize the therapeutic activity of tegafur, the first DPD inhibitory fluoropyrimidine, tegafur-uracil (UFT), was developed, and tegafur and the DPD inhibitor uracil were combined at a molecular ratio of 1:4, respectively [35]. The addition of uracil to tegafur has been shown to enhance the fluorouracil concentration in tumor tissues versus normal tissues. Ota *et al.* reported in the results of a Phase II study that UFT is well tolerated, with antitumor activity in a wide variety of solid tumors [36]. Daily oral administration of UFT and leucovorin achieved similar antitumor efficacy in colon cancer compared with intermittent intravenous administration of 5-FU and leucovorin [27,37]. UFT is now approved in over 50 countries as a cancer therapy, most commonly for advanced colorectal cancer, to replace 5-FU.

S-1 is oral fluoropyrimidine that combines the 5-FU prodrug, tegafur, a DPD inhibitor, gimeracil and an orotate phosphoribosyltransferase (OPRT) inhibitor, oteracil potassium (Oxo), at a molecular ratio of 1:0.4:1, respectively. It was developed in Japan by Shirasaka *et al.* [30]. Animal experiments suggest that Oxo is distributed at high levels in the digestive tract after oral administration, thereby relieving the gastrointestinal toxicity induced by 5-FU [38,39]. Thus, S-1 improves the selectivity of 5-FU action, prolongs the efficacious 5-FU concentration in the blood through its DPD inhibitor, gimeracil, and reduces toxicity through the OPRT inhibitor, Oxo [40]. A pharmacokinetic study of orally administered S-1 by Hirata *et al.* revealed that S-1 has a similar effect to continuous intravenous infusion of 5-FU [39]. S-1 is now approved in Japan as a monotherapy for a wide range of cancers, such as gastric, colorectal, pancreatic and biliary tract [41]. S-1 has also been approved in other Asian countries, including Korea, China, Singapore, Hong Kong, Taiwan and Thailand. S-1 combined with cisplatin had similar effects as infusion of 5-FU with cisplatin in the FLAGS trial and was approved for the treatment of advanced gastric or gastroesophageal cancer in the EU in 2011 [42].

Capecitabine (N4-pentyloxycarbonyl-5′-deoxy-5-fluorocytidine), an orally administered 5-FU pro-drug, has been already approved in > 100 countries including many European and Asian countries. It was rationally designed to generate 5-FU predominantly within tumor cells [43,44]. It is converted to 5-FU by three enzymes located in the liver and tumors. It can be metabolized to 5′-deoxy-5-fluorocytidine by carboxylesterases in the liver, converted to 5′-deoxy-5-fluorouridine (5′dFUR) by the cytidine deaminases in the liver and tumor tissue or converted into 5-FU by thymidine phosphorylase (TP), which is present in high concentration in tumors and their microenvironment. When combined, capecitabine can have antitumor effects once metabolized. As capecitabine is at least equivalent to 5-FU in terms of safety and efficacy, it can be used as a substitute for intravenous 5-FU [45]. Combination of capecitabine and oxaliplatin has been shown to be consistent with FOLFOX (oxaliplatin plus infusion of 5-FU and leucovorin) treatment for patients with metastatic colorectal cancer. Recently, Hong *et al.* showed that a combination of S-1 plus oxaliplatin is also consistent with a combination of capecitabine and oxaliplatin as first-line chemotherapy in patients with metastatic colorectal cancer [46]. These results indicate that the oral 5-FU prodrugs capecitabine and S-1 can be a substitute for infused 5-FU.

### 3.2 Thymidine analogs

TAS-102 is a novel oral nucleoside antineoplastic agent consisting of the thymidine analog, FTD, and a thymidine phosphorylase inhibitor (TPI) (5-chloro-6-(2-iminopyrrolidin-1-yl) methyl-2, 4 (1H, 3H)-pyrimidinedione hydrochloride), which inhibits degradation of FTD by TP in the liver [47,48]. FTD was first synthesized by Heidelberger *et al.* in 1964 [49]. This group demonstrated that FTD can be phosphorylated by thymidine kinase to its active monophosphate form [50]. Importantly, in preclinical studies and clinical trials, TAS-102 was active in 5-FU resistant tumors [47,51]. TAS-102 has several mechanisms of action (**Figure 3**) [52]. FTD incorporates into DNA and can inhibit TS to induce cytotoxicity [23,47,53]. Further, TPI enhances the bioavailability of FTD and can also inhibit angiogenesis [54,55]. TP, which is inhibited by TPI, was originally identified as a platelet-derived endothelial cell growth factor, which is present in high concentrations in tumors and their microenvironment [56]. As TPI inhibits the proliferation of endothelial cells, the secretion of antiangiogenic factors by cells with high TP expression, and TP-induced

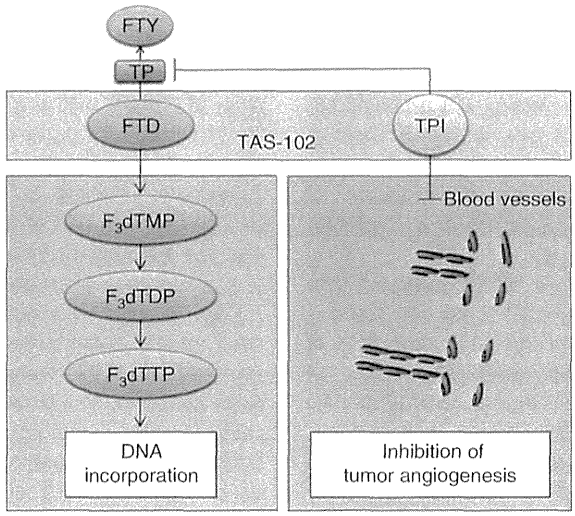


**Figure 3. The mechanism of TAS-102 antitumor action.** TAS-102 consists of FTD and a TPI. FTD is converted by thymidine kinase (TK) to its triphosphorylate form, F3dTTP, to incorporate into DNA and induce cytotoxicity. TPI inhibits thymidylate phosphorylase (TP) and suppresses the degradation of FTD to enhance the bioavailability of FTD. TPI also inhibits tumor angiogenesis, which is the proliferation of a network of blood cells that penetrates into cancerous growths, supplying nutrients and oxygen and removing waste products.

FTD: α,α,α-Trifluorothymidine; FTY: Trifluorothymine; F3dTDP: Trifluoromethyl deoxyuridine 5′-diphosphate; F3dTMP: Trifluoromethyl deoxyuridine 5′-monophosphate; F3dTTP: Trifluoromethyl deoxyuridine 5′-triphosphate; TP: Thymidylate phosphorylase; TPI: Thymidine phosphorylase inhibitor.



angiogenesis, TPI can be considered a potential antiangiogenic therapy [54,55,57]. The antitumor activity of TAS-102 in 5-FU-resistant tumors might be explained by the differences between FTD and 5-FU, as well as by the antiangiogenic effects of TPI.

In early clinical studies of FTD performed in the 1960s, different schedules of intravenous FTD administration were evaluated in patients with metastatic breast cancer and colorectal cancer. These studies showed that, although FTD had antitumor efficacy, it also causes severe myelosuppression and has a short elimination half-life. However, further clinical development of FTD has not been undertaken as the oral administration of FTD combined with TPI showed an improvement in the pharmacokinetic profile of FTD and the antitumor activity of FTD [48].

Several independent Phase I studies of patients with solid tumors were used to optimize TAS-102 dosage [58-62]. In 2012, Yoshino *et al.* presented evidence for the activity of TAS-102, as compared with placebo, for the treatment of patients with metastatic colorectal cancer who are refractory or intolerant to standard chemotherapy in a randomized comparative Phase II trial [51]. TAS-102 also appeared to be generally well tolerated. Importantly, these trials showed that the KRAS status may not directly affect the antineoplastic activity of TAS-102 because the mechanism of TAS-102 action involves direct incorporation of FTD into DNA. Based on the results of these clinical studies [51,62], TAS-102 was approved in Japan for the treatment of advanced metastatic colorectal cancer in March 2014. More recently, a global Phase III trial of TAS-102 in patients with refractory metastatic colorectal cancer met the primary efficacy end point of statistically significant improvement in overall survival versus placebo. The median overall survival time was 7.1 months (95% CI: 6.5 – 7.8) and 5.3 months (95% CI: 4.6 – 6.0) for TAS-102 and placebo-treated patients, respectively [63]. Future studies will help to delineate the mechanism of action of TAS-102 in tumors and the tumor microenvironment, and will identify biomarkers to predict those patients who would benefit most from treatment with TAS-102.

### 3.3 Cytidine analogs

Gemcitabine (2′,2′-difluoro 2′-deoxycytidine; dFdC) is an important cytidine analog for the treatment of gastrointestinal cancers, whereas other cytidine analogs, such as cytosine arabinoside (Ara-C), 5-azacytidine and 5-aza-2′-deoxycytidine (decitabine), are active in hematological malignancies. Gemcitabine was studied in a Phase I clinical and pharmacology trial in 1991, and has become an important drug for patients with several cancers, including pancreatic and non-small cell lung cancer [64,65]. The effect of gemcitabine is cell phase specific as it kills cells undergoing DNA synthesis and blocks the progression of cells through the boundary between the initial growth G1 phase and the S phase, in which DNA is synthesized [66].

Gemcitabine enters cells through the cell membrane via nucleoside transporters and is effectively accumulated in high concentrations in cells due to its relatively slow elimination half-life. Gemcitabine is converted intracellularly to the active metabolite, difluorodeoxycytidine, through a series of

sequential phosphorylations. In the first rate-limiting step, deoxycytidine kinase converts gemcitabine to gemcitabine monophosphate (dFdCMP). Subsequent phosphorylations lead to the accumulation of gemcitabine diphosphate (dFdCDP) and gemcitabine triphosphate (dFdCTP), which are both active metabolites. dFdCTP can interfere with DNA synthesis by competing with endogenous dCTP for incorporation into replicating DNA. In contrast, dFdCDP is a potent inhibitor of RR, which results in inhibition of deoxyribonucleotide triphosphate synthesis, specifically dATP. Importantly, gemcitabine could be a potent radiation sensitizer [67,68]. *In vivo* and *in vitro* studies have demonstrated that gemcitabine can enhance the antineoplastic activity of ionizing radiation in various cancer cells [68-71]. In addition, results from clinical trials suggest that gemcitabine functions as a radiosensitizer in patients [67]. Recently, Loehrer *et al.* demonstrated improved overall survival with the addition of radiation therapy to gemcitabine treatment in patients with localized unresectable pancreatic cancer, with acceptable toxicity [72].

### 3.4 Folic acid analogs

MTX (2,4-diamino-N10-methyl propylglutamic acid) is the most studied folate antagonist and is an effective therapeutic agent for many malignancies, as well as autoimmune diseases [73]. MTX acts as an inhibitor of dihydrofolate reductase, which is the enzyme required for the maintenance of the intracellular pool of THF. As THF and its metabolite, $CH_2THF$, are required for the synthesis of purines and thymidylate, MTX interrupts the synthesis of DNA and RNA, as well as other metabolic reactions.

## 4. Platinum analogs

Platinum-containing antineoplastic drugs are coordination complexes of platinum and have been widely used in the treatment of a variety of human cancers. The cytotoxic potential of platinum compounds was discovered in 1965 by Rosenberg *et al.* [74]. They cause the crosslinking of DNA through the formation of various adducts, including monoadducts, interstrand crosslinks, intrastrand crosslinks and DNA–protein crosslinks in cancer cells. Additionally, they interfere with the replication of DNA to stop the division of the cells and induce cytotoxicity [75]. Cisplatin is activated intracellularly through aquation of one of the two chloride groups, and subsequently covalently binds to DNA, forming DNA adducts. Clinical development of platinum analogs had been started, including cisplatin in the 1970s, carboplatin in the 1980s and oxaliplatin in the early 2000s.

### 4.1 Cisplatin

Cisplatin (*cis*-dichlorodiammineplatinum (II)) is the prototype of the platinum family of agents used to treat cancer. Cisplatin chemotherapy is curative in testicular cancer and is effective in lung, gynecological, gastrointestinal and genitourinary cancers, as well as cancers of the head and neck. For instance, in advanced biliary cancer, combination therapy of cisplatin and gemcitabine was associated with a significant survival advantage without the addition of substantial toxicity compared with gemcitabine alone, in Phase III trial [76]. Nevertheless, cisplatin has significant limitations. It is often necessary to discontinue cisplatin treatment because of adverse toxicities, such as nephrotoxicity, gastrointestinal toxicity such as nausea and vomiting, neurotoxicity, hematological toxicity and irreversible ototoxicity. Furthermore, cisplatin is effective only for a specific range of cancers.

Resistance to cisplatin can result from decreased accumulation, increased inactivation by cellular glutathione or an increased ability of the cells to tolerate cisplatin–DNA adducts [75,77-79]. Decreased accumulation of cisplatin is induced by a decrease in the active transport of the drug into cells through the copper transporter CTR1. Alternatively, accumulation can be inhibited through increased drug export from the cells through the copper exporters, ATP7A and ATP7B, and the glutathione S-conjugate export GS-X pump (MRP2 or ABCC2). The increased ability of cells to tolerate cisplatin-damaged DNA is induced by an increase in nucleotide excision repair and decrease in DNA mismatch-repair activity.

In addition to cisplatin, multiple platinum derivatives were tested in clinical trials. To date, only a few platinum analogs, such as carboplatin (*cis*-diammine-[1,1-cyclobutanedicarboxylato] platinum (II)) and oxaliplatin (1*R*,2*R*-diaminocyclohexane oxalatoplatinum (II)), have received worldwide approval for cancer therapy.

### 4.2 Carboplatin

Carboplatin has nearly the same range of clinical efficacy as cisplatin and is less toxic to the kidneys and more toxic to the bone marrow [75]. As the chloride groups in carboplatin have been changed, resulting in better delivery to cells and fewer side effects, it overcomes cisplatin-related toxicities, such as nephrotoxicity and neurotoxicity [80,81]. Early clinical studies with carboplatin reported that carboplatin is not nephrotoxic and reduces emesis compared with cisplatin [82]. In 1989, carboplatin was approved by the United States Food and Drug Administration (FDA) for ovarian cancer, and it has replaced cisplatin in the treatment of several malignancies. Recently, van Hagen *et al.* showed that preoperative chemotherapy with carboplatin, paclitaxel and radiation improved survival among patients with potentially curable esophageal or esophagogastric-junction cancer compared with surgery alone [6].

### 4.3 Oxaliplatin

Oxaliplatin has broader spectrum of antineoplastic activity than cisplatin and has, at least partially, overcome cisplatin resistance [83]. Oxaliplatin was first reported by Kidani *et al.*, who showed that adding different amino groups than found in cisplatin resulted in the formation of a bulkier DNA crosslink [84]. Oxaliplatin also showed a different sensitivity profile

than cisplatin in the NCI 60-cell human tumor panel [85]. Whereas cisplatin is effective in upper gastrointestinal malignancies, such as esophageal cancer and stomach cancer, oxaliplatin is much more effective in colorectal cancer. One reason for this difference is that the accumulation of oxaliplatin seems to be less dependent on CTR1. Further, mismatch repair recognition proteins do not recognize oxaliplatin-DNA adducts. Finally, differences between oxaliplatin-DNA adduct structures and cisplatin-DNA adduct structures may affect the cancers in which they are effective [75,86-88].

A 1992 clinical study of oxaliplatin with an infusion of 5-FU and leucovorin showed promising effects in patients with metastatic colorectal cancer [89]. In 2002, oxaliplatin was approved in the US for the treatment of colorectal cancer. Oxaliplatin, in combination with other anticancer agents, is currently the standard of care for advanced stage colorectal cancer. Although oxaliplatin has not been extensively studied in other malignancies and its range of effectiveness is fully unknown, it has recently been used for the treatment of pancreatic cancer. Conroy *et al.* showed that the combination chemotherapy regimen consisting of oxaliplatin with irinotecan and an infusion of 5-FU with leucovorin (FOLFIRINOX) was associated with survival advantage in patients with metastatic pancreatic cancer compared with the first-line therapy, gemcitabine [90].

### 4.4 NC-6004

Regimens including cisplatin are widely used for cancers, including gastric, lung, testicular, gynecological and genitourinary [75,91]. Currently, the use of targeted drug delivery systems (DDS) is being investigated for the specific accumulation of drugs in tumors [92]. This drug-targeting method is based on the principles of enhanced permeability and retention, and it is hoped it will lead to the development of antineoplastic drugs with greater therapeutic effects and fewer adverse effects [93]. In this approach, the drug accumulates in the tumor tissue by taking advantage of the pathophysiological characteristics of the tumor, including hyperplasia and hyperpermeability of tumor blood vessels. These characteristics can facilitate the extravasation of nanoparticles containing chemotherapeutic drugs. Importantly, because the nanoparticles are too large to pass though the smaller holes found in healthy tissue, they are less prone to leak from intact blood vessels.

NC-6004 (nanoplatin) is an innovative new drug containing cisplatin-incorporated micellar nanoparticles, which are composed of PEG-poly (glutamic acid) block co-polymers through a polymer–metal complex. NC-6004 is expected to reduce the drug toxicity of cisplatin and to increase antitumor efficacy. The basic nanotechnology of this formulation was invented by Kataoka and Nishiyama *et al.* [94,95]. Preclinical development of NC-6004 has been in progress in Japan [96]. Matsumura and Maeda demonstrated in 1986 that polymeric micelles containing cisplatin are preferentially distributed to tumors through the enhanced permeability and retention effect [97]. Further, Uchino *et al.* showed that NC-6004 had significantly lower toxicity than cisplatin and greater antitumor activity [98]. On the basis of these results, the first administration of NC-6004 in patients with advanced solid tumors in Phase I clinical study has been carried out in the UK [99]. A Phase I/II clinical study of NC-6004 in patients with advanced pancreatic cancer has been completed in Taiwan and Singapore. A Phase III study combining NC-6004 and gemcitabine for the treatment of advanced pancreatic cancer is also ongoing in Taiwan, Singapore, Hong Kong, China and Korea.

## 5. Conclusion

The recent decade has shown marked progress in how cancer is studied and how new therapies are developed [2,7,8]. However, despite advances in the treatment of cancer, including gastrointestinal malignancies, many patients still succumb to their disease due to drug resistance. In addition, many agents that were promising in preclinical studies fail to demonstrate similarly promising clinical activity as single agents in clinical trials. One of the major challenges on the road toward improved prognosis lies in the identification of combinations of novel molecularly targeted agents with conventional chemotherapy, including DNA synthesis inhibitors that overcome drug resistance. There is an urgent need for future clinical trials designed around novel combination therapies to achieve a higher response rate and longer remissions. To date, there are a vast number of laboratory, preclinical and clinical studies of DNA synthesis inhibitors, as well as novel molecularly targeted agents that hint for a synergistic approach. Efforts to examine patient samples from both tumors and healthy tissues are important to identify biomarkers to improve patient classification and, if possible, introduce personalized therapy for gastrointestinal cancers [3,100]. Translational cancer research to develop novel cancer therapeutics in gastrointestinal cancers will depend on close collaboration between basic researchers and clinicians, which will help to identify biomarkers, overcome drug resistance and improve the prognosis of patients and their quality of life.

## 6. Expert opinion

More than 50 years after the appearance of DNA synthesis inhibitors, such as antimetabolites, these drugs remain the most active category of anticancer drugs available and the standard therapeutics that new drugs are compared with. Although there has been a shift toward developing novel, rationally designed and specific therapeutics, the prognosis of gastrointestinal cancer still remains poor due to drug resistance. There are novel molecularly targeted agents in gastrointestinal cancers, including tyrosine kinase inhibitors such as imatinib, sunitinib, egorafenib, erlotinib, as well as therapeutic monoclonal antibodies such as bevacizumab, cetuximab and trastuzumab. Therefore, efforts to discover novel agents, as well as

novel chemotherapy combinations using molecularly targeted agents with conventional antineoplastic agents, have become increasingly important.

There are many challenges that must be overcome to identify novel DNA synthesis inhibitors. These include identifying ways to specifically and efficaciously target tumor cells, reducing chemotherapeutic toxicity, the development of biomarkers to predict pharmacological responses, rationally designing and testing combination therapies, and overcoming drug resistance.

The development of DDS is one method that could improve the low specificity of DNA synthesis inhibitors in cancer cells. There are two main concepts in DDS, either active targeting or passive targeting. Active targeting involves monoclonal antibodies or ligands to tumor-related receptors. Passive targeting systems can be achieved through enhanced permeability and retention effects. NC-6004, a cisplatin-incorporated polymeric micelle, is a promising drug using DDS technology in gastrointestinal cancer. A Phase III study evaluating the combination of NC-6004 and gemcitabine in advanced pancreatic cancer is ongoing.

The identification of biomarkers that define drug sensitivity, as well as drug toxicity, is a promising therapeutic strategy. Importantly, appropriate clinical trial designs are necessary in order to identify biomarkers to predict the clinical responses to new drugs. Phase I studies are needed to establish that the new drug inhibits the target molecule in the tumor. Phase II or III studies are required to obtain data for determining predictive biomarkers that will identify patients with tumors that are affected by the drug, thus allowing for the development of therapy-specific diagnostic tests. Efforts to examine patient samples from not only tumors, but also normal tissues, by various methods based on biochemistry, genetics, cytogenetics and epigenetics are important to identify biomarkers to improve patient classification and, if possible, introduce personalized therapy for gastrointestinal malignancies. Caution is needed against over reliance on the biomarker strategy to predict drug sensitivity as intratumor heterogeneity has been identified in various cancers, including gastrointestinal malignancies, and has important implications for acquired drug resistance.

The challenges to improved prognosis can be found in the identification of both promising therapeutic agents and combination therapies to overcome drug resistance. Translational cancer research will design novel combination therapies rationally in order to achieve a higher response rate and longer remissions.

Translational research to develop novel cancer therapeutics in gastrointestinal tumors will depend on close collaboration between basic researchers and clinicians, which will help to identify biomarkers, overcome drug resistance and improve the prognosis of the patients and their quality of life.

## Declaration of interest

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