ANSWERING REVIEWERS

March 1, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: Main manuscript_ WJG_8918.doc).

Title: *IGF2* DMR hypomethylation in relation to pathological and molecular features of serrated lesions

Author: Takafumi Naito, Katsuhiko Nosho, Miki Ito, Hisayoshi Igarashi, Kei Mitsuhashi, Shinji Yoshii, Hironori Aoki, Masafumi Nomura, Yasutaka Sukawa, Eiichiro Yamamoto, Yasushi Adachi, Hiroaki Takahashi, Masao Hosokawa, Masahiro Fujita, Toshinao Takenouchi, Reo Maruyama, Hiromu Suzuki, Yoshifumi Baba, Kohzoh Imai, Hiroyuki Yamamoto, Shuji Ogino, Yasuhisa Shinomura

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The manuscript has been improved according to the suggestions of reviewers:
1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer

We have responded to each of the comments as indicated below. Reviewers' comments are italicised, and in square brackets. Within the revised manuscript, changes to the text in response to the reviewers' comments are underlined, using colored text.

Reviewer (1)

[There are some criticisms to meet prior to accept it for publication. The authors should better specify the different categories of serrated polyps, namely the SSAs sessile serrated adenomas, as the literature report a architectural dysplasia in such polyps, not a cytological dysplasia as reported for TSAs traditional serrated adenomas.]

As requested, we have added the sentences in the Materials and Methods section (page 8, paragraph 2) as follows:

SSAs are characterized by the presence of a disorganized and distorted crypt growth pattern that is usually easily identifiable upon low-power microscopic examination. Crypts, particularly at the basal portion of the polyp, may appear architecturally distorted, dilated, and/or branched, particularly in the horizontal plane, which leads to the formation of boot, L, or anchor-shaped crypts. The cytology is typically quite bland, but a minor degree of nuclear atypia is allowable, particularly in the crypt bases [15, 25, 26].

[Abstract: "More then 100" please state exact numbers]

As requested, we have stated exact numbers in the Abstract section (page 4) as follows:

To accurately analyze the association between the histological types and molecular features of each type of serrated lesion, we consecutively collected 1386 formalin-fixed paraffin-embedded (FFPE) tissue specimens that comprised all histological types [hyperplastic polyps (HPs, N = 121), sessile serrated adenomas (SSAs, N = 132), traditional serrated adenomas (TSAs, N = 111), non-serrated adenomas (N = 195), and colorectal cancers (CRCs, N = 827)].

[Abstract: P ≤ 0.038 does it refer to DMR0 or LINE1?]

As requested, we have added *P*-values for *IGF2* DMR0 and LINE-1, respectively in the Abstract section (page 5) as follows:

The methylation levels of *IGF2* DMR0 and LINE-1 in TSAs with HGD (50.2 ± 18.7 and 55.7 ± 5.4, respectively) were significantly lower than those in TSAs (61.6 ± 19.6 and 58.8 ± 4.7, respectively) (*IGF2* DMR0; *P* = 0.038, LINE-1; *P* = 0.024).

[Introduction: "SSAs are hypothesized to develop to some populations of MSI-high CRCs with BRAF mutation in the proximal colon" please change the word populations]

As requested, we have changed the "populations" to "cases" in the Introduction section (page 6, paragraph 2) as follows:

Therefore, SSAs are hypothesized to develop in some cases to MSI-high CRCs with *BRAF* mutation in the proximal colon [7, 15, 17, 25, 26, 28, 29].

[In the introduction I would spend a sentence on the mechanism of carcinogenesis of DMR0/IGF2 While the rationale for Kras, BRAF and MSI testing is implicit in the introduction the rationale of examining IGF and PIK3 is not explained]

As requested, we have added the sentences in the Introduction section (page 6, paragraph 3 and page 7 paragraph 1, respectively) as follows:

With regard to the *PIK3CA* gene, a previous study reported that no mutation was found in serrated lesions, and that mutations were uncommonly, but exclusively, observed in non-serrated adenomas (1.4%) [30].

However, to date, there have been no studies describing the role of *IGF2* DMR0 hypomethylation in the early stage of colorectal carcinogenesis.

[Results: in table 2 I is unclear if the 120 SSA include dysplasia. I would put all the dysplasia cases in table 3]

I agree with the reviewer's comment. To avoid confusion, we revised "Sessile serrated adenoma (SSA)" to "Sessile serrated adenoma (SSA) without cytological dysplasia" in Table 2. Moreover, we have put cases of SSAs with cytological dysplasia in Table 3.

[In table 3 SSA are missing because they are reported in table 2 There is a large amount of data that is difficult to digest, the authors should use multivariate analysis where possible]

We thank this helpful comment. A multivariate logistic regression analysis was employed to examine associations with *IGF2* DMR0 hypomethylation (as an outcome variable), adjusting for potential confounders. The model initially included sex, age, tumor size, tumor location, histological type, and the LINE-1 methylation level, and MSI, *BRAF*, *KRAS*, and *PIK3CA* mutations. Our data showed that the *IGF2* DMR0 hypomethylation was inversely associated with SSAs ($P < 0.0001$) (page 11, paragraph 3).

[It is unclear how DMR0 hypometilation correlates with MSI, Kras, BRAF and PIK3, specifically was DMR0 hypometylation correlated with Kras mutations?]

Previously we reported that multivariate analysis on 1105 colorectal cancers has revealed that tumor *IGF2* DMR0 hypomethylation was independently associated with LINE-1 hypomethylation, but not significantly with MSI, *BRAF*, *KRAS*, or *PIK3CA* mutation (Baba Y, Nosho K et al. Gastroenterology 2010).

Likewise, using serrated lesions and non-serrated adenomas, we examined whether the *IGF2* DMR0 hypomethylation was correlated with those molecular alterations. As a result, *IGF2* DMR0 hypomethylation was associated with LINE-1 hypomethylation, but not significantly with MSI, *BRAF*, *KRAS*, or *PIK3CA* mutation.

[Discussion: "Therefore, our data challenge the common conception of discrete molecular features of SSAs versus 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"= I do not understand, since the SSA had different levels of methylation compared to TSA and HP, why the authors say that their findings challenge the concept of different molecular features among serrated lesions]

I agree with the reviewer's comments. Therefore, we have deleted the sentences "Therefore, our data challenge the common conception of discrete molecular features of SSAs versus 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".

[As the authors state, the conclusions on TSA high grade dysplasia (and SSA HGD) are based on few samples, TSA per se was not correlated with hypometylation so the results are very preliminary. Finally, given the high percentage of CRC cases with DMR0 hypometylation how do the authors reconcile this with the rarity of TSA?]

We have shown that *IGF2* DMR0 hypomethylation frequently occurred in non-serrated adenomas with HGD, as well as TSAs with HGD. Therefore, many CRCs with *IGF2* DMR0 hypomethylation may arise from not only TSAs with HGD but also non-serrated adenomas with HGD.

[DISCUSSION The authors write: "IGF2 DMRO hypomethylation was less frequently detected in SSAs compared with HPs TSAs and non serrated adenoma" . The interaction between hyperplastic HPs microvescicular and SSAs should be better clarified. Passages and non return point of IGF2 DMRO between HPs and SSAs should be better detailed.]

Our data have shown that *IGF2* DMR0 methylation levels of SSA were higher than those of microvescicular HP, suggesting that *IGF2* DMR0 methylation level may affect the progression of HP. In other words, HPs with *IGF2* DMR0 hypomethylation may tend not to progress to the typical SSA pathway but to the alternate pathway.

Therefore, we have added the sentences in the Discussion section (page 16, paragraph 3) as follows:

<u>These results imply that *IGF2* DMR0 hypomethylation may be a key epigenetic event that affects the progression of HPs</u>.

Reviewer (2)

[This is an original article which is based on a huge analyzed data, indicating the role of IGF2 DM0 hypomethylation in the progression of serrated tumors related to malignant transformation. It should be determined that it is worthy to be published in this journal.]

We appreciate the reviewer's comment.

3 References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely,
Katsuhiko Nosho
Department of Gastroenterology, Rheumatology and Clinical Immunology,
Sapporo Medical University School of Medicine, Sapporo, Japan
S-1, W-16, Chou-ku, Sapporo, 060-8543 Japan
TEL: +81 (11) 611-2111, FAX: +81 (11) 611-2282
E-mail: nosho@sapmed.ac.jp