Author's response to reviews

Title: DNA methylation alterations of AXIN2 in serrated adenomas and colon carcinomas with microsatellite instability

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Author's response to reviews: see over
Dear Dr. Julie Cunningham
Associate Editor, BMC Cancer

Thank you very much for your letter dated March 26 2014, with the detailed and constructive comments from the two reviewers on our submitted manuscript entitled “DNA methylation alterations of AXIN2 in serrated adenomas and colon carcinomas with microsatellite instability” by Yuta Muto, Takafumi Maeda, Koichi Suzuki, Takaharu Kato, Fumiaki Watanabe, Hidenori Kamiyama, Masaaki Saito, Kei Koizumi, Yuichiro Miyaki, Fumio Konishi, Sergio Alonso, Toshiki Rikiyama and myself.

We have made an effort to revise the manuscript to incorporate the reviewers’ comments, and have amended the manuscript accordingly. The answers to all the comments by the reviewers are described one by one in the following pages of this cover letter. Each modified point is underlined. As a couple of the referees’ comments are shared, we address these comments jointly.

I trust the revised paper will satisfy the referees and editor.

Sincerely yours,

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Reviewer: Keith Robertson

Previous research has demonstrated genetic and epigenetic alterations in sessile serrated adenomas (SSA) and colon carcinomas with (MSI) (Maeda T, et al, 2011). The mechanism by which adenoma transitions to MSI remains unknown. In this paper the authors analyzed that genome-wide surveillance of hyper- and hypo-methylation alterations in NotI sites by MSFLP-array in tubular adenoma (TA), SSA, colon carcinomas with (MSI) and without microsatellite instability (MSS). The authors show that 1) between these four groups, 56 probes were differentially altered in analysis of DNA methylation. 2) These four groups fell into two clusters: Group 1, TAs and MSS cancers with KRAS mutations. Group 2 was composed of the cells with BRAF mutations. Group 2 was further divided into two subgroups; cancer with MSI and MHL1 mutation (Group 2A) and SSAs without MHL1 mutation (Group 2B). 3) The authors also revealed that aberrant methylation of AXIN2 is observed in SSA and MSI. From these results, the authors suggest that repression of AXIN2 by DNA methylation might trigger a transition of SSA adenoma cells with BRAF mutation, into MSI cancers.

We thank the reviewer for his comments. We would like to correct the review in that the stratification entails MLH1 methylation, and not mutation. Furthermore, we only suggested in the paper, as a working hypothesis, that Axin 2 silencing may contribute to the transition of SSA to MSI cancer, not to trigger the transition.

The authors describe convincing results but there are several concerns.

Major point

1) The authors categorized genetic and epigenetic changes of TA, SSA, MSI and MSS. The categorized data is very similar to their own previously reported data (Maeda T, et al, 2011). This is most likely because the authors used same specimens that they had used in previous reports (Maeda T, et al, 2011). So there is some question as to how much these new findings add to our understanding of SSAs and their progression.

As the reviewer mentions, genetic and epigenetic features of TA, SSA, MSI and MSS were categorized in our previous study, by analysis of a handful of specific selected genes that had been already characterized. In this study we validated our previous findings in a subset of the samples by a comprehensive analysis using unbiased 9,654 DNA fragments on MS-AFLP arrays, and we showed somatic methylation alterations of many loci, including AXIN 2. We also show methylation profiles sharing between SSA and MSI. There are many additional loci that have been discovered in our study that could be characterized in more detail in subsequent studies, which is not the case with the Maeda et al. paper.
2) The authors have shown for the first time that aberrant DNA methylation of AXIN2 in SSA and the methylation status was apparently increased in MSI. From these results, the authors hypothesized silencing of AXIN2 might be a trigger of transition of SSA adenoma cells with BRAF mutation, into MSI. But, the author’s group previously reported that AXIN2 transcripts were suppressed in most of MSI colorectal carcinoma specimens. (Koinuma T, et al, 2006). Therefore while the finding of AXIN methylation is new, it is not unexpected given the expression patterns already reported.

Koinuma and colleagues were in the same Jichi University, but in a different research group. As the reviewer points out, Koinuma K. et al. reported that AXIN2 transcripts were suppressed in most of MSI cancers, but never mentioned about the change of AXIN2 transcripts in their precursor, SSA. In this study, we demonstrate that AXIN2 transcripts were gradually suppressed by methylation not only in MSI cancers, but also in SSA. In response to reviewer’s suggestion, we revised the manuscript in discussion as follows.

Our results suggest that expanding of methylation in the promoter region of AXIN2 in SSAs lead to the suppression of the AXIN2 gene expression gradually, which contributes to a stepwise acquisition of the epigenetic features seen in MSI colon cancer. Koinuma T et al reported that overexpression of AXIN2, either by treatment with 5’-azacytidine or by transfection with AXIN2 cDNA, resulted in rapid cell death in a MSI CRC cell line, which supports the functional significance of AXIN2 changes in methylation and expression in our study.

Minor point
1) The numbers in the table 1 and 2 are difficult to understand. The numbers should be written as a ratio. For example, in table 1, KRAS mutation in TA should be written as 4/8 (mutant/total). hMLH1 methylation in TA also should be written as 0/8 (methylation +/total).

We have changed the tables 1 and 2 as suggested by the reviewer.
Reviewer: Subbaya Subramanian

Reviewer's report:

*Muto et al, describes the analyses of DNA methylation in sessile serrated adenomas (SSA) and colorectal cancers with microsatellite instability (MSI). The authors used an array-based methylation sensitive amplified fragment length polymorphism method to analyze the genome-wide methylations status of SSA and MSI tissue cancer samples. The authors show that there were no major hypermethylation differences between or within benign and malignant tumors groups regardless of their clinical and genomic parameters. Further, hypomethylation was also found to be less frequent in SSAs compared with MSI or MSS samples. They also conclude that AXIN2 gene had more methylation alterations and hypermethylation of MLH1, when occurs in an adenoma cell with BRAF mutation, drives MSI cancer.

Major essential revisions.

The sample size used in this study seems to be too low to achieve statistical significance.

We thank the reviewer for his comments. We do not completely agree with the reviewer’s opinion. The sample size in this study was sufficient for finding differences statistically significant. The P values are provided in the Tables and Figure 1. For instance, SSAs were younger (P=0.019) and had less hypomethylation (P=5.8x10^-4) than MSI cancers (Table 1). However, we agree that the number of samples was small to reach definitive conclusions in some other comparisons. In response to the reviewer’s suggestion, we revised the manuscript in discussion as follows.

In conclusion, this study revealed that methylation aberrations likely play a role in the serrated adenoma-MSI carcinoma sequence in colon cancer. Although the samples in this study are too limited to draw definitive conclusions in some genetic or epigenetic comparisons, other differences were sufficiently large to reach statistical significance. MLH1 silencing seems to occur in an already developed serrated adenoma by the previous occurrence of somatic mutation in the BRAF oncogene.

Further, the functional characterization of loss or gain-of-function of AXIN in colon cancer cells will strengthen the conclusion of this study.

The functional characterization of loss or gain-of-function of AXIN in colon cancer cells would strengthen the conclusions of this study. However, we do not believe these studies are...
required in the present report, because they would represent an enormous and time consuming experimental task and because the conclusions reached were independent of functional validation. Moreover, Koinuma T et al demonstrated that forced expression of AXIN2, either by treatment with 5′-azacytidine or by transfection with AXIN2 cDNA, resulted in rapid cell death in a MSI CRC cell line. (Koinuma K, et al: Epigenetic silencing of AXIN2 in colorectal carcinoma with microsatellite instability. Oncogene 2006, 25:139-146).

In response to reviewer’s suggestion, we revised the manuscript in the discussion as follows.

Our results suggest that expanding of methylation in the promoter region of AXIN2 in SSAs lead to the suppression of the AXIN2 gene expression gradually, which contributes to a stepwise acquisition of the epigenetic features seen in MSI colon cancer. Koinuma T et al reported that overexpression of AXIN2, either by treatment with 5′-azacytidine or by transfection with AXIN2 cDNA, resulted in rapid cell death in a MSI CRC cell line, which supports the functional significance of AXIN2 changes in methylation and expression in our study.

Additional experimental validations are required substantiate their claim that “hypermethylation of MLH1, when occurs in an adenoma cell with BRAF mutation, drives MSI cancer”

We do not completely disagree with the referee. However, we explicitly stated that “We conclude that hypermethylation of MLH1, when occurs in an adenoma cell with BRAF oncogenic mutationial activation, drives the pathway for MSI cancer by providing the cells with a mutator phenotype”

So, it is our interpretation of the results and as such it is not meant as a firm statement that would need additional experimental validations. The conclusion is based on several assumptions and facts disclosed by this study.

1. First, we show for the first time that MLH1 is not hypermethylated in SSAs, which therefore are not MSI, despite having already a mutated B-raf oncogene. Therefore, B-raf mutation precedes MLH1 hypermethylation and silencing and MSI. Since MSI cancers contain B-raf mutations and MSI is due to MLH1 silencing, hypermethylation of MLH1 must occur in cells already having B-raf mutations.
2. Second, we assume that B-raf activation is what induces transformation of a precursor cell to an adenoma cell. At least, it is an important factor for the transformation. There are many studies showing the role of oncogene activation (i.e. K-ras) in transformation, especially in the adenoma stage.

3. Third, it is well established that once a mutator phenotype due to mismatch repair deficiency unfolds, this drives tumorigenesis through a specific pathway that involves the accumulation of many mutations in a particular subset of cancer genes. The process may take several years, which is one of the reasons why HNPCC cancers develop around ten years later than those of the FAP syndrome. APC mutation is already critical for cell growth deregulation (adenomagenesis) while MLH1 inactivation only causes a strong mutator phenotype that does not accelerates the growth of the cells.

What is not yet clear is what originates the B-raf mutations - if they are not just spontaneous -, and MLH1 methylation, but once these events occur the pathway towards cancer is already set.