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Title: Loss of CDX2 expression is associated with poor prognosis in colorectal cancer patients

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

1. Revision has been made according to the suggestions of the reviewers.

(1) Novel finding of this manuscript: Clinicopathologic characteristics of decrease or loss of CDX2 expression was investigated by many researchers. However, independent prognostic value of loss of CDX2 expression was not clearly demonstrated yet. In this study, we presented that loss of CDX2 expression has independently poor prognostic value adjusting TNM stage and other potential confounders (p.6 row 7-12, p.16 row 6-12)

(2) Strength and weaknesses of this study: We evaluated CDX2 expression using two different anti-CDX2 primary antibodies (CDX-88 and EPR2764Y) in 713 surgically resected CRCs (p.7 row 22 – p.8 row 1). To our knowledge, this study is the largest comprehensive study focusing CDX2 expression in CRCs in East Asia, and the third largest study in the world. But, this study has some limitations. First, proportion of CIMP-high, MSI-high and *BRAF* mutation in this study were lower than those of Western population. Second, rectal cancers were underrepresented because we excluded patients who received preoperative chemotherapy and/or radiotherapy. Third, we evaluated CDX2 expression in single core tissue microarray in each case. Fourth, ROC-curve based determination of immunohistochemical analysis could be a source of overfitting to clinicopathologic and survival analysis (p.16 row 13 – p.17 row 4).

(3) Data extraction: In revised manuscript, we added 38 CRC cases which were missed in the original manuscript but fulfilled inclusion criteria (p.7 row 2 – 4). Clinical data were collected by review of electric medical record system by Bae JM. These data were checked by Kang GH. Microscopic examination was performed by Bae JM and Kang GH without knowledge of molecular study results (p.7 row 8 – 12). Molecular studies were performed by Lee TH and Cho NY.

(4) Terminology: We corrected CIMP-H,L and MSI-H,L to CIMP-high, CIMP-low, MSI-high and MSI-low. Decreased CDX2 expression is changed to loss of CDX2 expression.

(5) Study for microsatellite instability: Genomic DNA of normal mucosa and tumor were extracted in each case. MSI status was analyzed using 5 NIH marker panel by high-performance liquid chromatography (HPLC) (p.8 row 16-18).

(6) Validation of CDX2 expression in immunohistochemistry: In our original manuscript, we measured CDX2 expression status using clone CDX2-88 which is formerly widely used anti-CDX2 primary antibody. But, false negativity of CDX2-88 immunoreactivity in low-CDX2 expressing tumors is reported. So, we validated CDX2 expression status using newly introduced anti-CDX2 primary antibody (EPR2764Y) (p.7 row 22 – p.8 row 1). Using cut-off of <20% of nuclear positivity, Two antibodies showed tolerable agreement (p.10 row 10 – p.11 row 4). Data using CDX2-88 were used in the revised manuscript, but clinicopathologic, molecular and survival data showed similar results between CDX2-88 and EPR2764Y. Cut-off for loss of CDX2 expression varies among studies. Dawson et al. used cut-off of <20% of nuclear stain to correlate loss of CDX2 expression with methylation of CDX2. But, they used <90% for MMR-deficiency, ≤25% for CIMP and *BRAF* mutation (Dawson H., International Journal of Cancer, 2013).

(7) Survival analysis: In our original manuscript, we included all possible potential confounders in multivariate analysis, and insignificant variables were eliminated by backward elimination method. In revised manuscript, we performed multivariate analysis using variables which showed statistical significance in univariate analysis (p.9 row 17 – 20). In original manuscript, we used disease-free survival. In revised manuscript, progression-free survival was used to show prognostic value in stage IV CRCs. In our original manuscript, adjuvant chemotherapy was an independent prognostic factor in multivariate analysis. But, adjuvant chemotherapy itself had insignificant prognostic value in univariate survival analysis. So, we didn't include adjuvant chemotherapy in multivariate analysis.

(8) Citation of recently published articles: Our original manuscript didn't mention some recently published articles. In the revised manuscript, we cited two original articles (Dawson et al's "Possible role of Cdx2 in the serrated pathway of colorectal cancer characterized by *BRAF* mutation, high-level CpG Island methylator phenotype and mismatch repair-deficiency" in International Journal of Cancer, 2013 and Tsai et al's "Aberrant expression of annexin A10 is closely related to gastric phenotype in serrated pathway to colorectal carcinoma", Modern Pathology, 2014) (p.15 row 15-17, p.16 row 4-6), and Olsen et al's qualitative review ("The clinical perspectives of CDX2 expression in colorectal cancer: A qualitative systematic review" in Surgical Oncology, 2014) (p.14 row 18-21).

(9) Summary of expression of immunohistochemical markers: In our original manuscript, we used box and whisker plot to present expression of immunohistochemical markers. But, Expression of CK7, Ck20 and CDX2 showed skewed distribution. So, we used histogram in revised manuscript. Mean and standard deviation of each marker was described in the revised manuscript (Figure 2 and Supplementary Figure 1).

(10) Explanation for poor clinical outcome of loss of CDX2 expression: Recently, loss of CDX2 expression has been considered in the context of the serrated neoplasia pathway or gastric phenotype of CRCs, which is associated with poor clinical outcome (p.15 row 9 – 17). Although CIMP-high, MSI-high and *BRAF* mutation were not independent prognostic factor in this study, we think that poor clinical outcome of loss of CDX2 expression originated from additive effects of those molecular features.

2. Explanation for what reviewers mentioned, but we couldn't include in the revised manuscript.

(1) Clinicopathologic characteristics of excluded patients: Among 989 consecutively resected CRCs, 276 cases were excluded. We did not collect clinical data of those patients. And we couldn't collect them during revision period regretfully.

(2) Cytoplasmic stain of CDX2: In embryonal stage, expression of CDX2 is detected in both nucleus and cytoplasm (Silberg DG, Gastroenterology, 2000). In normal colonic mucosa of adult and premalignant lesions, CDX2 expression is detected in nucleus. But, cytoplasmic stain is reported in CRCs and tumors in other organs (Ishikawa A, Laboratory Investigation, 2004; Bakaris S, Histol Histopathol, 2008). Some investigators consider cytoplasmic stain of CDX2 as nonspecific crossreactivity or non-functional fraction of CDX2, but there might be an unknown function of cytoplasmic CDX2 in tumors. In this manuscript, we measured only nuclear stain of CDX2, because cytoplasmic expression of CDX2 is out of scope of this manuscript.

(2) Molecular classification: Jass JR. proposed five molecular subtypes based on CIMP and MSI status (Jass JR, Histopathology, 2007). After then, several molecular classification systems were proposed by other investigators. We mentioned about correlation between molecular subtypes and loss of CDX2 expression in the original manuscript, but we eliminated that result in the revised manuscript to focus clinical implication of loss of CDX2 expression.

(3) CDX2 expression in metastatic tumors: CDX2 expression in metastatic lymph nodes or distant organs is a kind of interest. But, this topic is out of scope in our manuscript.

Sincerely,

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