Correlations of ALD, Keap-1, and FoxO4 expression with traditional tumor markers and clinicopathological characteristics in colorectal carcinoma

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Abstract
Aldolase A (A-2) (ALD), Kelch-like-ECH associated protein-1 (Keap-1), and Forkhead box O4 (FoxO4) are key regulatory proteins, which have been proven to be involved in tumor development. However, the clinicopathological significance of ALD, Keap-1, and FoxO4 expressions in colorectal (colon) carcinoma (CRC) is not clearly known. We sought to explore the clinicopathological significance of ALD, Keap-1, and FoxO4 in CRC to provide evidences for potential monitoring index of CRC. Cases of 199 CRC patients were analyzed retrospectively. Evaluation of ALD, cAMP response element-binding protein-2, cyclo-oxygenase-2, FoxO4, Keap-1, and p53 expressions in CRC patients was accomplished with immunohistochemical technique. The patients were divided into negative and positive groups in accordance with immunohistochemical result. We compared the clinicopathological characteristics of the patients in the 2 groups, coupled with analysis of the relationship between 6 aforesaid proteins and clinicopathological characteristics. Herein, we confirmed the association of tumor location with the expression of ALD, Keap-1, and FoxO4. Also, tumor differentiation was observed to associate significantly with the expression of Keap-1, FoxO4, and Cox-2. The data also revealed that there was a correlation between smoking and expression of ALD, Keap-1, FoxO4, p53, and Cox-2. Nevertheless, insignificant difference was observed when clinicopathological characteristics were compared with cAMP response element-binding protein-2 expression. These findings suggest that ALD, Keap-1, and FoxO4 involved in CRC development, and thus may be considered as potential monitoring index for CRC.

Abbreviations: ALD = aldolase A(A-2), CD = Crohn disease, Cox-2 = cyclo-oxygenase-2, CRC = colorectal carcinoma, Creb-2 = cAMP response element-binding protein-2, FoxO4 = Forkhead box O4, HIF-1α = hypoxia inducible factor-1 alpha, Keap-1 = Kelch-like-ECH associated protein, VEGF = vascular endothelial growth factor.

Keywords: ALD, colorectal carcinoma, FoxO4, Keap-1

1. Introduction
Among the various cancer types, the most common diagnosed tumor is colorectal carcinoma (CRC), which is mainly associated with increased mortality around the world.[1] Chronic inflammation is implicated in the pathological process of CRC, wherein Crohn disease (CD) or ulcerative colitis, the 2 main types of inflammatory bowel diseases are the culprits.[2] Markers for early diagnosis, monitoring during treatment, and prognostic prediction have not been discussed clearly. To improve the prognosis of CRC patients, it is necessary to find potential proteins for monitoring.

Tumor progression or suppression occurs as a result of multiple proteins’ functions. As an enzyme of glycolysis, aldolase A (A-2) (ALD) is considered as a tumor promoter for regulating the epithelial-mesenchymal transition and associated signaling pathways in CRC.[3,4] Available literature has suggested the importance of ALD in cells’ proliferation and tumor formation in hypoxic conditions.[5] According to previous studies, ALD was established to be downstream target gene of hypoxia-inducible factor 1-alpha (HIF-1α).[6,7] Glycolytic pathway activity was found to associate with the expression of ALD.[8] As a multi-subunit protein, cullin-3-based Cullin-RING E3-ubiquitin ligase is discovered to comprise...
Keap-1 (Keap-1). The complex often exerts regulatory effects by constituting the signaling pathway with Nrf2, which is important regulator in redox homeostasis in normal tissues and can also promote cell proliferation and survival in cancers. On the one hand, the Keap-1-Nrf2 pathway is crucial to survival of cells coupled with defense against oxidative stress and xenobiotics, which protects healthy cells against carcinogenesis. Meanwhile, epigenetic modifications or somatic mutations in Keap-1 can lead to the accumulation of Nrf2 in tumor cells, which consequently promotes proliferation and resistance of cancer cells to drugs. Interestingly, ALD was also observed to be regulated by Nrf2 amid contribution to radio-resistance in the stem cells of breast cancer. In addition, Forkhead box O4 (FoxO4) has been reported to possess the capacity to stimulate as stated above, DAB (2040A0925; Beijing Zhongshan Gold Bridge Biotechnology Co., Ltd., Beijing, China) was added as chromogen. Hematoxylin was used to counterstain the sections.

The detailed information of antibodies used in this work can be seen in Table 1. We mounted thick sections (4 µm) on microscope slides after they have been cut from blocks of paraffin. Later, we stained them via immunohistochemical streptavidin-peroxidase method. Afterwards, deparaffinization of the sections was carried out in xylene before rehydration with graded ethanol. We heated the slides for 2 minutes in buffer solution of citrate (pH6) with microwave after PBS washing, before 30 seconds exposure to 100°C for antigen retrieval. Later, we cooled them at room temperature and washed as stated above. Activity inhibition of endogenous peroxidase was carried out with hydrogen peroxide (3%). We added primary antibodies after washing as described above, while the entire sections were incubated at 4°C overnight. Addition of secondary antibodies and incubation of the sections were performed after they have been rinsed in PBS. After rinsing as stated above, DAB (2040A0925; Beijing Zhongshan Gold Bridge Biotechnology Co., Ltd., Beijing, China) was added as chromogen. Hematoxylin was used to counterstain the sections. Negative control was prepared by replacing primary antibody with PBS. The slides were examined under the microscope. The detailed information of antibodies used in this work can be seen in Table 1.

2.3. Hematoxylin-eosin staining

The scores of staining were evaluated with semi-quantitative scoring system under light microscope by a trained observer without knowing the outcome and other clinical determinations of the patients. We firstly scanned the slides at 10x magnification and then scored them at higher magnification. The following rule of immunostaining intensity scoring was used, namely no stain = 0, weak stain = 1, moderate stain = 2, and strong stain = 3. Grading of positive cells percentage in tumors was as follows: 1 = 11% to 20%, 2 = 21% to 30%, 3 = 31% to 50%, 4 = 51% to 75%, and 5 = 76% to 100%. The eventual scores were estimated by multiplying both, while positive cell was evaluated if the score was more than 2.

Table 1

| Product name                        | Manufacturer       | Lot.  | Dilution |
|-------------------------------------|--------------------|-------|----------|
| Aldolase A Antibody (A-2)           | Santa Cruz Biotechnology | sc-377058 | 1:200 |
| Anti-FOXO4/AFX antibody             | Abcam (UK)         | ab126757 | 1:200 |
| Keap1 Antibody (G-2)                | Santa Cruz Biotechnology | sc-365626 | 1:200 |

2.5. Assessment of immunohistochemistry

We included 199 CRC patients who were diagnosed of CRC between December 2019 and September 2020, including 87 female and 112 male. Tumor stages were evaluated according to guidelines (8th edition) supplied by American Joint Committee on Cancer. Evaluation of differentiation grades was accomplished based on laid-down guidelines provided by the World Health Organization. Consents of patients were sought prior to collection of their tissue samples for diagnosis and research. Approval of the study was issued by the ethics committee at Jiangsu University, while performance of the experiments followed guidelines of Helsinki declaration.

2.2. Recruitment of patients and collection of specimens

We included 199 patients who were diagnosed of CRC at Jiangsu University Hospital between December 2019 and September 2020, including 87 female and 112 male. Tumor stages were evaluated according to guidelines (8th edition) supplied by American Joint Committee on Cancer. Evaluation of differentiation grades was accomplished based on laid-down guidelines provided by the World Health Organization. Consents of patients were sought prior to collection of their tissue samples for diagnosis and research. Approval of the study was issued by the ethics committee at Jiangsu University, while performance of the experiments followed guidelines of Helsinki declaration.

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2.6. Statistical analysis

SPSS 26.0 (IBM, Chicago, Illinois, USA) was used for the analysis of statistics. Number of cases and constituent ratio were used to express the enumeration data. The measurement data were expressed as the mean ± standard deviation values. We evaluated the association of various clinicopathological characteristics (e.g., tumor differentiation, tumor location, Duke stages, etc) with the expression of ALD, Keap-1, FoxO4, p53, Cox-2, and Creb-2 with chi-square tests. Statistically, consideration was given to \( P < .05 \) as acceptable significant level.

3. Results

3.1. FoxO4, ALD, and Keap-1 expression in CRC tissues

Figure 1 shows the tumor tissues at diverse differentiation stages. According to the figures, the cells in the tumor with poorer differentiation were disorder with hyperchromatic nuclei, while the glandular cavities were not discovered. Figure 2 displays the expression of FoxO4, ALD, and Keap-1 in CRC. In CRC cells, the main location of positive FoxO4 and Keap-1 expression was cytoplasm (Fig. 2A–F), however,
ALD was expressed in both cytoplasm and nuclei (Fig. 2B and E).

3.2. The characteristics of patients

Patients (199) with 170 (85.4%) beyond 50 years were analyzed in our study, wherein they comprise 87 (43.7%) females and 112 (56.3%) males. Specifically, 7 (3.5%) had their tumors located in the ileocecal, 36 (18.1%) in the colon ascendens, 5 (2.5%) in the colon transversum, 7 (3.5%) in the colon descendens, 44 (22.1%) in the colon sigmoideum, and 100 (50.3%) in the colon sigmoideum rectum. Out of 199 overall cases, 1 (0.5%), 8 (4.0%), 163 (81.9%), 10 (5.0%), and 17 (8.6%) cases, respectively, demonstrated well, moderately-moderate, low to medium, and poor differentiation. In terms of Duke stage, 2.5% of the patients were placed in stage A, 54.3% in stage B, 38.7% in stage C, and 4.5% in stage D. In these cases, 8.5% of the patients had existed Schistosoma infection, 29.1% of them exhibited smoking habit with 59.8% showing alcoholic habit. Notably, p53 was expressed in 64.3% of the patients, Ki67 in 94%, 5-Fu in 58.3%, Cox-2 in 37.8%, and Cerb-2 in 30.7%. The clinicopathological characteristics of the 199 CRC patients are summarized in Table 2.

3.3. Relationship of ALD, Keap-1, FoxO4, p53, Cox-2, and Cerb-2 with clinicopathological characteristics

Upon statistical analysis, ALD expression in CRC was found to associate with sex ($P < .001$), tumor location ($P < .001$), infection by Schistosoma ($P = .002$), and smoking ($P < .001$). The tumor of the patients with positive expressing ALD tended to locate in colon ascendens. Also, Keap-1 expression significantly correlated with tumor location ($P = .04$), differentiation ($P < .001$), and smoking ($P < .001$). The data revealed that among the Keap-1 expression positive group, colorectal tumor may more likely be located in colon transversum. Moreover, we observed association of positive FoxO4 expression in CRC with tumor location ($P < .001$), differentiation ($P < .001$), and smoking ($P < .001$). The tumor of patients in FoxO4 expression positive group can possibly be located in the colon descendens. Additionally, the correlation between the expressions of these proteins was also examined. Our analysis displayed that the expressions of ALD ($P = .04$) and Keap-1 ($P = .006$) were related to that of p53. Also, the expression of 5-Fu showed a reciprocal correlation with those of Keap-1 ($P = .009$) and FoxO4 ($P = .001$). Likewise, to FoxO4 expression in the patients, that of Cerb-2 significantly increased ($P = .009$). The results also revealed a significant relationship between p53 expression and smoking ($P < .001$). Besides, we found that ALD, Keap-1, FoxO4, and p53 expressions tended to be negative in the patients who were smokers. Cox-2 was more likely to be detected in CRC who demonstrated smoking ($P < .001$) or alcohol ($P < .001$) habit, while no significant relationship was observed between Cerb-2 expression and the aforementioned clinicopathological characteristics. The detailed data and analysis are shown in Tables 3 and 4.

4. Discussion

Herein, we observed significant association between tumor location and expressions of ALD, Keap-1, and FoxO4. The tumor in positive ALD expression group was more likely to be located in the colon ascendens. The location of tumor in positive Keap-1 expression group tended to be colon transversum, while in positive FoxO4 expression, tumor was possibly located in the colon descendens. The correlation between tumor differentiation and expression of Keap-1 and FoxO4 was also discovered.

According to previous studies, overexpression of ALD and Keap-1 in several cancer types has been reported.[4,28,36] ALD can promote tumor growth by regulating glycolytic pathway. Ye et.al observed the correlation of ALD expression with clinical stage, tumor invasion depths, and tumor location,
which is similar with ours.\textsuperscript{4} Furthermore, FoxO4 can inhibit expression of glucose transporter-1, erythropoietin, and vascular endothelial growth factor, which is crucial in tumor expansion and tumor progression.\textsuperscript{37} Sun et al.\textsuperscript{38} also showed that FoxO4 overexpression substantially decreased CRC cells migration and in vivo metastasis. Consistent with

| Characteristics | ALD |  | Keap-1 |  | FoxO4 |  |
|-----------------|-----|-----|-------|-----|-------|-----|
|                 | +   | -   | $P$ value | +   | -   | $P$ value | +   | -   | $P$ value |
| Sex             |     |     |         |     |     |         |     |     |         |
| Male            | 62  | 50  | $<.0001$ | 37  | 75  | .2389   | 56  | 56  | 1.0000  |
| Female          | 72  | 15  | .3033    | 36  | 51  | 43      | 44  |     |         |
| Age, yr         |     |     |         |     |     |         |     |     |         |
| ≤50             | 17  | 12  | $<.0001$ | 11  | 18  | 1.0000  | 13  | 16  | 1.0000  |
| >50             | 116 | 54  | .0914    | 62  | 108 | 75      | 95  |     |         |
| Location        |     |     |         |     |     |         |     |     |         |
| Ileocecal       | 0   | 7   | $<.0001$ | 1   | 6   | $.0389  | 0   | 7   | $<.0001$ |
| Colon ascendens | 35  | 1   | .9938    | 17  | 19  | 16      | 20  |     |         |
| Colon transversum | 0  | 5   | .4482    | 4   | 1   | 1       | 4   |     |         |
| Colon descendens | 7  | 0   | .3331    | 1   | 6   | 5       | 2   |     |         |
| Colon sigmoideum | 36 | 8   | .3331    | 14  | 30  | 7       | 37  |     |         |
| Rectum          | 52  | 48  | .3331    | 28  | 72  | 64      | 36  |     |         |
| Differentiation |     |     |         |     |     |         |     |     |         |
| Well differentiation | 1  | 0   | .9938    | 0   | 1   | 0       | 1   |     |         |
| Moderately-well differentiation | 6  | 2   | .4482    | 7   | 1   | 6       | 2   |     |         |
| Moderately differentiated | 102 | 61  | .3331    | 54  | 109 | $.0914  | 83  | 80  | $<.0001$ |
| Low to medium differentiated | 9   | 1   | .3331    | 4   | 6   | .0914   | 10  | 0   | $.0914  |
| Poorly differentiated | 15 | 2   | .3331    | 16  | 1   | .0914   | 1   | 16  | $.0914  |
| Dukes stages    |     |     |         |     |     |         |     |     |         |
| A               | 3   | 2   | .9938    | 2   | 3   | $.9441  | 2   | 3   | .9745   |
| B               | 71  | 37  | .4482    | 36  | 72  | 53      | 40  |     |         |
| C               | 50  | 27  | .3331    | 25  | 52  | 37      | 5   |     |         |
| D               | 6   | 3   | .3331    | 3   | 6   | 4       | 5   |     |         |
| Infection by Schistosoma |     |     |         |     |     |         |     |     |         |
| Yes             | 5   | 12  | $.0020   | 5   | 12  | $.6087  | 5   | 12  | $.1262  |
| No              | 127 | 55  | .4482    | 67  | 115 | 94      | 88  |     |         |
| Smoking         |     |     |         |     |     |         |     |     |         |
| Yes             | 30  | 28  | $.001    | 12  | 46  | $.001   | 18  | 40  | $.001   |
| No              | 119 | 22  | .4482    | 76  | 65  | .988    | 98  | 43  | .988    |
| Alcohol         |     |     |         |     |     |         |     |     |         |
| Yes             | 81  | 38  | .3331    | 49  | 70  | .3331   | 59  | 60  | 1.0000  |
| No              | 50  | 30  | .3331    | 20  | 40  | .3331   | 40  | 40  | .3331   |
| p53 expression  |     |     |         |     |     |         |     |     |         |
| Yes             | 83  | 45  | $.0360   | 58  | 70  | $.0062  | 70  | 58  | $.6570  |
| No              | 35  | 36  | .5862    | 18  | 53  | .365    | 36  | 35  | .365    |
| Ki67 expression |     |     |         |     |     |         |     |     |         |
| Yes             | 122 | 65  | $.1242   | 72  | 115 | $.2257  | 101 | 86  | 1.0000  |
| No              | 5   | 7   | $.2257   | 7   | 5   | .65    | 6   | 6   | .65    |
| 5-Fu expression |     |     |         |     |     |         |     |     |         |
| Yes             | 73  | 43  | $.2871   | 43  | 73  | $.0091  | 55  | 61  | $.0013  |
| No              | 59  | 24  | .3647    | 36  | 47  | .592    | 59  | 24  | .592    |
| Cox-2 expression|     |     |         |     |     |         |     |     |         |
| Yes             | 86  | 34  | $.2857   | 38  | 77  | $.1822  | 58  | 57  | $.3894  |
| No              | 54  | 30  | .2857    | 36  | 48  | .48     | 48  | 36  | .48     |
| Creb-2 expression |     |     |         |     |     |         |     |     |         |
| Yes             | 41  | 20  | $.7489   | 27  | 34  | $.2697  | 41  | 20  | $.0093  |
| No              | 89  | 49  | .7489    | 49  | 89  | .65     | 65  | 73  | .65     |

The bold means that $P$ value is less than .05. ALD = aldolase A(A-2), FoxO4 = Forkhead box 04, Keap-1 = Kelch-like-ECH associated protein-1.
these results, we also found that the expression of ALD, Keap-1, and FoxO4 were significantly associated with tumor location.

It was reported that Keap-1 can induce the cancer cell proliferation and malignant progression as a transcriptional in the Keap-1-Nrf2 pathway, which may lead to lower
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risk of CRC postoperative survival via survival and prognostic relationship of ALD, Keap-1, and FoxO4 expressions with the prospective and well-designed studies are needed to affirm the merit for CRC diagnosis by policy makers. Furthermore, larger populations. In clinical guidelines, the expression of ALD, Keap-1, and FoxO4 should be considered as part of the risk assessment for CRC diagnosis by policy makers. Furthermore, larger prospective and well-designed studies are needed to affirm the relationship of ALD, Keap-1, and FoxO4 expressions with the risk of CRC postoperative survival via survival and prognostic analyses.

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Author contributions

PH, ZZ, and GS conceived and designed the experiments. YZ, AJ, and CR conducted the experiments. SW, MK, ZW, XQ, and XW collected the samples and analyze the data. SW, MK, and ZW drafted the manuscript, which have been read and approved by all the authors for publication in this current form.

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