Material and methods

This is a selected retrospective study included 50 different cases of colorectal adenocarcinoma designated as; 38 cases of conventional adenocarcinoma, 6 cases of mucinous adenocarcinoma and 6 cases of signet ring cell carcinoma (all cases were right or left colectomy specimens). Six control cases were taken from viable margins in patients with intestinal infarction. Sections of normal liver tissue (adjacent liver tissue to cholecystectomy specimen) were taken as a positive control for ALDH1A1 immunohistochemical expression.

The material included archival formalin fixed paraffin embedded blocks processed during the years 2015-2020 as well as stained Hematoxylin and Eosin (H&E) slides for review. The blocks were collected from Pathology Department and Early Cancer Detection Unit; Faculty of Medicine, Benha University, Egypt. Clinicopathological data were collected from the files of patients. Being a retrospective study, a written informed consent was not needed. The study was approved by the Research Ethical committee of Faculty of Medicine, Benha University, Egypt.

Histopathological examination:

Re-evaluation of sections from all selected cases, unaware of their diagnosis, was performed. The cases were re-evaluated for their subtype and graded into well differentiated, moderately differentiated, and poorly differentiated tumors **(Sato et al., 2010; Awan et al., 2017).** Lymph node status was evaluated and TNM staging system was applied to the cases according to AJCC, 8^{th} edition **(Amin et al., 2017; Nagtegaal et al., 2019).**

Immunohistochemical studies:

For immunohistochemical studies, Aldehyde dehydrogenase 1A1 (ALDH1A1) antibody staining was performed for all biopsies, using Avidin-Biotin complex technique to detect presence of tumor cells, through their cytoplasmic expression for such immunohistochemical marker. Steps were as follow;

1- Formalin-fixed, paraffin embedded tissue sections were cut at 4 micrometer thickness and mounted on positively charged slides (StarFrost slides, Waldemar Knittel Glasbearbeitungs GmbH, Braunschweig, Germany).

2- Slides were deparaffinized in 2 changes of xylene (10 minutes each) then rehydrated through descending grades of ethyl alcohol (100%, 95% and 70%).

3- Slides were washed with distilled water three times for 2 minutes each.

4- For antigen retrieval:

a) Slides were placed in a Koplin jar containing solution of 10 mmol/L citrate monohydrate buffer (pH 6.0). Koplin jar was placed in a water bath to keep a humid atmosphere inside the microwave oven.

b) Slides were placed in microwave oven at 800 WATT power for 3 cycles, 5 minutes each. The amount of fluid in koplin jar was checked and water was added if necessary to prevent slides from drying out.

c) The jar was removed from the oven and allowed to cool for 20 minutes at room temperature.

5- Blocking endogenous peroxidase activity was done by immersing the slides in 3% hydrogen peroxide in 30% methanol for 15 minutes. Then sections were washed with distilled water to stop peroxidase activity.

6- Sections were incubated with the diluted primary Rabbit polyclonal antibody (1:50) for ALDH1A1 ***(Chongqing biopsies co., Cat No YPA1390, China, conc)*** overnight.

7- Antibody-binding was detected by use of a standard labeled streptavidin-biotin system ***(Genemed, CA 94080, USA, South San Francisco)***, it was incubated with slides for 20 minutes then washed with distilled water.

8- Freshly prepared chromogen diaminobenzine (DAB, Envision ^{TM} Flex /HRP-Dako, REF K 8000) was used; it was incubated with slides for 15 minutes then washed with distilled water.

9- Slides were counter stained for 3 minutes with Mayer's hematoxylin (BioGrenex cat. No. 94583).

10- Slides were rinsed in water, dehydrated in ascending grades of alcohol 70%, 95%, 100% then cleared in xylene.

11- A drop of DPX (Distyrene, Plasticizer and Xylene) wash mountant was added and sections were covered by a glass cover**.**

Positive control:

A section of normal liver tissue was used as external positive control **(Li et al., 2012; Kalantari et al., 2017).**

Negative control:

Tissue section was processed in the above mentioned sequence with omitting the primary antibody and adding Phosphate Buffered Saline (PBS) instead **(Wang et al., 2013; Kalantari et al., 2017).**

Interpretation of ALDH1A1 expression:

Positivity was considered as brownish homogenous cytoplasmic staining of tumor cells **(Li et al., 2014; van der Waals et al., 2018).**

The immunohistochemical scores were obtained by light microscopy as the staining intensity (scored from 0–3) multiplied by the percentage area of positive immunostaining within the visual field (the percentage of positive cells within 5 high power fields in hot areas) (scored from 0–4). The intensity of ALDH1A1 protein expression was scored as: 0 (no staining); 1 (weak staining); 2 (moderate staining); or 3 (strong staining). The percentage area of positive immunostaining was scored as: 0 (<5%); 1 (5–25%); 2 (26–50%); 3 (51–75%); or 4 (>75%) **(Yang et al., 2018).**

The cut-off value for high versus low expression of the ALDH1A1 protein was determined using receiver-operating characteristic (ROC) curve analysis and SPSS statistical software, defining a final immunostaining score of >3.5 as high ALDH1A1 protein expression **(Yang et al., 2018) (figure 9, table 21).**

Statistical analysis:

The clinico-pathological data were recorded on a report form. These data were tabulated and analyzed using the computer program SPSS (Statistical package for social science) version 25 (SPSS Inc., Chicago, IL, USA) to obtain:

Descriptive data:

Descriptive statistics were calculated for the data in the form of:

1. Mean and standard deviation $(\pm SD)$. for quantitative data.
2. Frequency and distribution for qualitative data.

Analytical statistics:

In the statistical comparison between the different groups, the significance of difference was tested using:

- Inter-group comparison of categorical data, performed by using chi square test (X^2-value) and fisher exact test (FET).

$$x^2 = \frac{\sum (observed - \exp ected)^2}{Expected}$$

$$Expected = \frac{col.total \ x \ row total}{Grand \ total}$$

- A *P* value < 0.05 was considered statistically significant (*), while > 0.05 statistically insignificant. P value < 0.01 was considered highly significant (**) in all analyses.