EFFECT OF HELICOBACTER PYLORI INFECTION ON CANCER STEM CELL MARKER (CD133) IN COLORECTAL CANCER

Mayadah Mohammed Ridha Abdul-Razzaq¹, Zainab Fadhel Ashoor², Wasan Abdul-ilah Bakir³
Department of Microbiology / College of Medicine / Al-Mustansiriya University ², ³, Department of Microbiology / Iraqi Center of Cancer and Medical Genetic Research / Al-Mustansiriya University, Iraq.
*Corresponding author’s email: maiada_life2000@yahoo.com

ABSTRACT
Human CD133 gene consists of at least 37 exons is located in chromosome 4, and its length is about 152 kb and it used for the identification and isolation of cancer stem cell population from malignant tumors as specified in colon. Prominin is cell surface antigen that known as a possibility CSC marker in colon. H. pylori is gram negative spiral shape, may be associated with colorectal cancer. The aim of this study was to assess the expressions of CD133 and H. pylori in colorectal cancer tissue by using immunohistochemical staining and to analyze the clinical significance of the expressions related to other clinicopathological data and survival results. Paraffin- embedded tumor tissue of specimens from (30) cases with colorectal cancer tissues and (30) colorectal normal tissues were assessed by immunohistochemistry for the expression of CD133 & H. pylori. There was highly statistically significant difference among the extent of scores both of CD133 & H. pylori staining in colorectal cancer and normal controls. Regarding the association between grades of differentiation the result show highly statistical significance expression of CD133; but the result show no statistical significance expression of Anti H. pylori IHC scores between grade of differentiation for colorectal cancer. Regardless of its significance as a CSC marker; however, our results suggest that evaluation of CD133 staining might be useful to identify colon cancer patients at high risk of recurrence and death and CD133 increases expression with infection of H. pylori.

KEYWORDS: CD133, H. pylori, Colorectal cancer.

INTRODUCTION
Colorectal cancer is the third commonly diagnosed cancer in males and the second in females, with 1.4 million cases and almost 694,000 deaths predestined to have occurred in 2012¹. Cancer of the colorectal collectively can also be called cancer of the large intestine or large bowel; it is most common malignant tumor in the advanced world.² In Iraqi according to the (IRC) the colorectal cancer represented about (5.36%) of all malignant tumors registered during the period from 2011in female 4.36% (476 cases) and in male 6.52% (610 cases)³. A recent study ratified the idea that (CRC) pathogenesis might be specific cell surface markers for cancer stem cells (CSCs) are needed for identifying and sorting the CSCs. Several markers for CSCs have been investigated in colorectal cancer CD133 have been the most frequently researched and are thought to be the most likely markers for colorectal CSCs.⁴. These cells are sometimes called “stem cells or tumor-initiating cells” and several markers have been found to be expressed in these cell populations.⁵ Their ability of self-renewal unbounded proliferation, and multipotency are considered cancer stem-cell phenotypes, and they responsible for local relapse and metastasis by stimulating resistance against traditional drug therapy.⁶ Highly resistant to chemotherapy and radiotherapy which makes them very hard to target and eliminates them by common therapy regimens, this characteristic makes them a possible source of later repetition of the disease chosen of resistant clones⁶. CD133 was identified for the first time in 1997 on normal human hematopoietic stem cells⁷. It is PROMAL1 or prominin is cell surface antigen that has newly been recognized as a possibility CSC marker in brain, colon and prostate cancer.⁸. A structural model of CD133 showed that this protein is characterized by an extracellular N-terminus, a cytoplasmic C-terminus, 2 small cysteine rich cytoplasmic loops and 2 very large extracellular loops each containing 4 potential sites for N-linked glycosylation⁹,10. Human CD133 gene consisting of at least 37 exons is located in chromosome 4, and its length is about 152 kb ¹¹. H. pylori is Gram-negative spiral-shaped bacterium¹². Within geographical areas, the prevalence of H. pylori inversely correlates with socioeconomic status; in particular in relation to living conditions during childhood.¹³. The prevalence of H. pylori in industrialized countries generally remains under 40% and is considerably lower in children and adolescents than in adults and elderly people.¹⁴. It has 2 to 6 unipolar, which often carry a distinctive bulb at the end; the flagella confer motility and allow rapid movement in viscous solutions such as the mucus layer overlying the gastric epithelial cells; it lacks fimbria adhesions¹⁵. It is microaerophilic growth requirements with optimal growth at O₂ levels of 2 to 5% and need of 5 to 10% CO₂ and high humidity there is no need for H₂O₂. growth occurs at 34 to 40°C with an optimum of 37°C, the bacterium will survive at pH range of (5.5 to 8).¹⁶ The aims of the Study to identify the expression of certain CSCs like CD133 and improve their useful use for identification of colorectal cancer and to investigate the
possible increased prevalence of _H. Pylori_ infection in the preneoplastic condition of the colon that include adenoma.

**MATERIALS & METHODS**

Thirty patients with colorectal adenocarcinoma (mean age years and range), the control group include 30 colorectal normal mucosa with mean age and range between were involved in this study. Biopsy specimens were collected from the archive of the department of histopathology of teaching laboratories of Al-Yarmook Teaching hospital and Educational laboratories of medical city for the period between 2015 and 2016 and informed consent was obtained from all patients. Biopsies were fixed in 10% formal buffer saline for histological examination. The biopsies were used for histological evaluation and immunological staining for CD133 monoclonal antibody and _H. pylori_ polyclonal antibody. Tissue sections cut into 4 µm thickness, put on positively charged slides.

Mucosal biopsies were immune staining with primary antibody: anti-CD133 monoclonal antibody (Mybiosource / USA Cat# MBS 850595, 1: 100, Anti- _H. pylori_ Rabbit polyclonal to _H. pylori_ Microorganism Abcame / UK code (ab20459) 1:100). The use of mouse and rabbit Specific HRP/DAB Detection IHC Kit. The primary antibody reacts with antigen in the tissue, and then a biotin labeled secondary antibody (link antibody) binds to the primary antibody. When the conjugate is added, the biotinylated secondary anti-body will form a complex with the peroxidase-conjugated streptavidin and by adding the substrate, which contains 3,3diaminobenzidine (DAB) in a chromogen solution, a brown-colored precipitate will form at the antigen site. In the peroxidase secondary detection system, the presence of a brown reaction product at the site of the target antigen is indicative of positive reactivity. Counter stain will be pale to dark blue coloration of the cell. Evaluation of the immunostaining was done with the assistance of a histopathologist. The observer was blinded to the clinical diagnosis of the tissues at the time of assessment, and tissues were independently assessed by two observer positive or negative cases, positive immunostaining gave dark brown granules. In the peroxidase secondary detection system, the presence of a brown reaction product at the site of the target antigen is indicative of positive reactivity. Counter stain will be pale to dark blue coloration of the cell.

**Scoring**

<table>
<thead>
<tr>
<th>TABLE 1: Explicate type and number of tissue in each group</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>I</td>
</tr>
<tr>
<td>II</td>
</tr>
<tr>
<td><strong>Total</strong></td>
</tr>
</tbody>
</table>

**Grade of differentiation for colorectal cancer**

Thirty colorectal cancer tissue samples were collected during a period of the study and analyzed by histopathological examination identifying tumor types and grades, as described under (materials and methods). Grade of differentiation for colorectal cancer found 2(6.7%) cases of well differentiated, 22(73.3%) cases of moderate differentiated, which is the largest group, and only 1(3.3%) case of poorly differentiated, while 5(16.7%) cases of in situ carcinoma. The results are shown in (figure 1).
Frequency of CD133 marker IHC scores in the study subjects
Figure 2, revealed CD133 protein was expressed in all colorectal cancer tissue samples 30(100%), moderately expression were the most frequent scores among total cases 18(60%) followed by strong expression 10(33.3%), and only 2(6.7%) are weakly expressed. While the most frequent scores of CD133 expression in normal control colon tissue were weakly expressed 16(53.3%), and 11(36.7%) moderate and there were 3(10%) showed no expression. There was highly statistical significant difference among the extent of scores CD133 staining in colorectal cancer and normal controls using Pearson Chi-square test at 0.05 levels (P value = 0.0001).

The Frequency of Anti H. pylori marker IHC scores in the study subjects
Figure 3, revealed that H. pylori protein was positive expression in colorectal cancer tissue samples 17(56.7%), while H. pylori positive expression (infected with H. pylori) in normal controls appeared 7 (23.3%), and H. pylori negative expression (not infected with H. pylori) 13 (43.3%) and 23 (76.7%) in colorectal cancer and normal controls tissue samples respectively, all result it can be concluded that positive expression revealed the highest percentage of expression among groups in colorectal cancer (56.7%) compared to the healthy controls appeared highest percentage of expression (76.7%). There was highly statistical significance in the extent of scores H. pylori staining in colorectal cancer and normal controls.
**H. Pylori** infection on cancer stem cell marker (CD133) in colorectal cancer

Frequency of CD133 IHC scores in the study subjects according to grade of differentiation:

CD133 protein was expressed in all of colorectal cancer tissue samples with positive expression being the most frequent scores among both moderately differentiated malignancy, well differentiated malignancy and in situ carcinoma 14(63.6%), 1(50%) and 3(60%) respectively, with strong expression among both moderately differentiated malignancy 8 (36.4%), well differentiated malignancy 1(50%) and poorly differentiated malignancy 1(100%). For weak expression of CD133 only appeared in situ carcinoma 2(40%), all results can be concluded that moderate expression revealed the highest percentage of expression among grade of differentiated in colorectal cancer, compared to other levels. Regarding the association between grade of differentiation the result show highly statistical significance expression of CD133 by using Pearson Chi-square test at 0.05 levels (P value=0.008). The result showed in table (3).

**TABLE 2: CD133 IHC scores among studied group according to grade of differentiation**

<table>
<thead>
<tr>
<th>Colorectal carcinoma</th>
<th>Grade of differentiation</th>
<th>No</th>
<th>%</th>
<th>No</th>
<th>%</th>
<th>No</th>
<th>%</th>
<th>No</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>scores of CD133</td>
<td>No expression</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Weak (1-10%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>40.0</td>
</tr>
<tr>
<td></td>
<td>Moderate (11-50%)</td>
<td>1</td>
<td>50.0</td>
<td>14</td>
<td>63.6</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>60.0</td>
</tr>
<tr>
<td></td>
<td>Strong (&gt;50%)</td>
<td>1</td>
<td>50.0</td>
<td>8</td>
<td>36.4</td>
<td>1</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.031*</td>
</tr>
</tbody>
</table>

*Significant difference between proportions using Pearson Chi-square test at 0.05 level.

Frequency of H. pylori IHC scores in the study subjects according to grade of differentiation:

*H. pylori* was positive expression in colorectal cancer tissue samples with positive expression being the most frequent scores in moderately differentiated malignancy 14 (63.6%), well differentiated malignancy poorly differentiated malignancy and in situ carcinoma 1(50%) and 1(100%) respectively. *H. pylori* not infected among both well, moderately and poorly differentiated malignancy 1(50%), 8(36.4%) and 4(80%) respectively, all results can be concluded that positive staining revealed the highest percentage of expression among grade of differentiation in colorectal cancer, compared to negative staining. Regarding the association between grade of differentiation, the result show no statistical significance expression of Anti H. pylori IHC scores by using Pearson Chi-square test at 0.05 levels (P value=0.264). The result showed in table (4).

**TABLE 3: Anti H. pylori IHC scores among studied group according to grade of differentiation**

<table>
<thead>
<tr>
<th>Colorectal carcinoma</th>
<th>Grade of differentiation</th>
<th>No</th>
<th>%</th>
<th>No</th>
<th>%</th>
<th>No</th>
<th>%</th>
<th>No</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>scores of Anti H. pylori</td>
<td>Negative (No staining)</td>
<td>1</td>
<td>50.0</td>
<td>8</td>
<td>36.4</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>80.0</td>
</tr>
<tr>
<td></td>
<td>Positive (with staining)</td>
<td>1</td>
<td>50.0</td>
<td>14</td>
<td>63.6</td>
<td>1</td>
<td>100</td>
<td>1</td>
<td>20.0</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.264</td>
</tr>
</tbody>
</table>

*Significant difference between proportions using Pearson Chi-square test at 0.05 level.

The statistical analysis of CD133 in colorectal cancer and normal controls immunoexpression and the difference in its expression among different studied subjects:

There was highly statistical significant difference in mean level of CD133 protein expression between colorectal cancer and normal controls tissue samples by using Students-t-test at 0.05 levels. (P value=0.0001).

**FIGURE 4:** Box plot showed the differences in mean of CD133 expression between colorectal cancer and normal controls by using Students-t-test.

The statistical analysis of *H. pylori* in colorectal cancer and normal controls immunoexpression and the difference in its expression among different studied subjects:
There was highly statistical significant difference in mean level of Anti-\( H.\) \( pylori \) protein expression between colorectal cancer and normal controls tissue samples by using Students-t-test at 0.05 level (\( P \) value=0.0001).

**FIGURE 5:** Box plot showed the differences in mean of Anti \( H.\) \( pylori \) expression between colorectal cancer and normal controls by using Students-t-test.

**Statistical analysis of CD133 and infection with \( H.\) \( pylori \) in colorectal cancer and normal controls:**

Table 4.19, revealed an association between CD133 and infection with \( H.\) \( pylori \). CD133 showed expression in a moderate and strong level, with moderate and strong expression of CD133 appeared \( 7(38.9\%) \), \( 10(100\%) \) respectively, infected with \( H.\) \( pylori \) in colorectal cancer, and in normal controls \( 7(63.6\%) \) moderate expression of CD133. While weak expression of CD133 \( 2(100\%) \) not infected with \( H.\) \( pylori \), moderate expression of CD133 \( 11(61.1\%) \) not infected with \( H.\) \( pylori \) in colorectal cancer. In normal controls appeared 23 cases not infected with \( H.\) \( pylori \), \( 3(100\%), 16 \) (100\%), 4(36.4\%) with no expression, weak and moderate expression of CD133 respectively. There was increased difference between CD133 and \( H.\) \( pylori \) (0.002) in colorectal cancer, and in normal controls, there was significance difference between CD133 and \( H.\) \( pylori \) (0.0001) by using Pearson Chi-square test at 0.05 level.

**TABLE 4:** Statistical analysis of CD133 with positive and negative staining of \( H.\) \( pylori \) in colorectal cancer and normal controls

<table>
<thead>
<tr>
<th>Scores of CD133</th>
<th>Colorectal carcinoma</th>
<th>Normal controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Scores of CD133</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No expression</td>
<td>Moderate (&gt;50%)</td>
</tr>
<tr>
<td></td>
<td>Weak (1-10%)</td>
<td>Moderate (&gt;50%)</td>
</tr>
<tr>
<td>Infected with ( H.) ( pylori ) (Anti ( H.) ( pylori )&gt;=5)</td>
<td>No</td>
<td>%</td>
</tr>
<tr>
<td>Infected</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Not (&lt;5)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Significant difference between proportions using Pearson Chi-square test at 0.05 level

**FIGURE 6:** Immunohistochemical staining for CD133 in colorectal carcinoma tissue, positive cytoplasmic (blue arrows). (X100)
Colorectal carcinoma was 4.89% of whole body malignancy and it is the seventh causes of death from cancer registered in Iraq [20].

**Expression of IHC staining of CD133 in all study subjects**

CD133 is a protein which is, also known as Prominin-1 or AC133, a cell surface transmembrane glycoprotein, was identified in subpopulations of cells in colon tumors [21]. Most of the colorectal carcinoma stem cell function as adhesion molecule with a role in colony forming, tumor invasiveness, differentiation, and survival [22]. However, in our results, CD133 was detected by using an immunohistochemical technique, it is expressed in all colorectal cancer tissue samples, moderately expression were the most frequent scores among total cases (60%), while the most frequent scores of CD133 expression in normal control colon tissue were weakly expressed (53.3%), there was highly statistical significant difference in mean level of CD133 protein expression between colorectal cancer and normal controls tissue samples with (P value=0.0001), this revealed that CD133 has been reported to be a cancer stem cell marker in colorectal cancer [4,21] and although some doubts have been arisen about its ability to specifically identify tumor initiating cells it has been widely used to identify and analyze cancer stem cell in colorectal cancer [23]. We were able to detect CD133 staining in the majority (63.3%) by comparison with other studies, which find, agreement of colon cancers analyzed although with a high heterogeneity in term of percentage of positive cells, whose increase was associated with an increased risk of recurrence and death for disease. These cells after routine chemotherapy or radiotherapy may lead to tumor recurrence and metastasis [24]. Furthermore, using tissue microarrays, failed to demonstrate an association between CD133 expression and tumor progression [25]. Some studies had showed that the expression of CD133 was not only in CSC but also in normal tissues and thought that CD133 might play a critical role in tumorigenesis [26].

Regarding the association between grade of differentiation the result show highly statistical significance expression of CD133 with moderate expression being the most frequent score of moderately differentiated malignancy, (63.6%) cases. The difference in results between poorly differentiated, well and moderately differentiated tumors are inconsistent with reports that poorly differentiated CRC is responsible for more extensive invasiveness [27] and poorer prognosis [28]. Our study disagreement with previous study [29], which find no significant of CD133 expression predicted a poor prognosis for colorectal cancer patients. Similarly, colorectal cancer may also include heterogeneous subtypes with and without CD133 positive cells with stem cell-like properties. Alternatively, the pathogenesis of poorly differentiated adenocarcinoma might not depend on CD133 positive cell population; Therefore, although immunohistochemical staining does not allow demonstration of self-renewal or the tumorigenic potential of CD133 positive cells, our findings suggested the need to further analyze associations between expression of (CIC) markers and clinicopathological features [30]. Alternation from cytoplasmic to membranous expression of CD133 was correlated to transition of epithelial cells to more invasive phenotype [31]. In our study, using IHC monoclonal and polyclonal antibodies detected CD133 expression at cytoplasm.

**Expression of IHC staining of *H. pylori* in all study subjects**

*Helicobacter pylori* are a common pathogen and class I carcinogen giving rise to gastric adenocarcinoma [32]. Experimental studies have demonstrated a relationship between certain *Helicobacter* species with inflammatory bowel disease and colonic adenocarcinoma development [33]. *H. pylori* infections have been considered as a risk factor for development of colorectal neoplasms (CRNs) such as colon polyps and colorectal cancer (CC) colorectal cancer due to the high prevalence of serologically positive *H. pylori* infection among CRN cases in some studies [34].

*H. pylori* was positive expression revealed the highest percentage of expression among groups in colorectal cancer (56.7%) compared to the healthy controls appeared highest percentage of expression (76.7%). In this result, there was highly statistical significant difference in mean level of Anti- *H. pylori* protein expression between colorectal cancer and normal controls tissue samples (P value= 0.0001), in contrast to other studies, the proportion of cases exhibiting positive and negative epithelial staining did not differ significantly with respect to *H. pylori* status [35]. The potential mechanism of the significant association between colon polyp and serologic *H. pylori* positivity has
been attributed to the remote trophic effect of the elevated gastrin level on the colonic mucosa[36]. The way in which \textit{H. pylori} interact with such host and environmental factors in the lumen of the large bowel to produce neoplasia remains unknown[37]. The host response induces epithelial cell proliferation through hypergastrinemia, which is reportedly associated with mitogenic effects on colorectal mucosa and with an increased risk of colonic malignancy in a subpopulation of patients[38]. Previous study designed to investigate an association between \textit{H. pylori} and extra gastric intestinal neoplasms and colonization in cases of direct colonic dysplasia has been investigated by specific IHC methods and they did not yield any correlation with colonic localization or histopathologic type. They were able to determine \textit{H. pylori} expression in colon polyps by immunohistochemical methods, albeit with no statistical significance[39]. \textit{H. pylori} was positive expression in colorectal cancer tissue samples with positive expression being the most frequent scores in moderately differentiated malignancy (63.6%). Our results, that positive staining revealed the highest percentage of expression among grade of differentiation in colorectal cancer, compared to negative staining. There was no statistical significance expression of Anti \textit{H. pylori}. As it comes to colorectal cancer, hypergastrinemia has been reported to be associated with increased risk for colorectal malignancy, as the hormone is considered to serve as a growth factor of normal colonic epithelium[39]. It is considered that the increased risk of colonic neoplasia in cases infected with \textit{H. pylori} is associated with the rise of production of gastrin triggered by these bacteria[40]. some studies which found, no statistical association between \textit{H. pylori} and colorectal neoplasm was found, but \textit{H. pylori} may increase the risk of adenoma[41]. However, no correlation between \textit{H. pylori} positivity and colon cancer could be demonstrated[42]. There exist serologic and colon tissue PCR studies that support[43] or reject[42], a correlation between \textit{H. pylori} and (CRNs). Our experience showed that with the proper design of PCR, a single copy of genomic DNA is sufficient to obtain a positive signal indicating the presence of \textit{H. pylori} in the sample[43].

**RECOMMENDATION**

CD133 and \textit{H. pylori} would be of great importance to fully understand the biology of individual proteins used as markers, because it can provide a new point of view on the seemingly contradictory results from individual studies. cancer stem cell markers can bring valuable information to patients' prognosis and can help to modify diagnostic and treatment strategy.

**REFERENCES**


Infection on cancer stem cell marker (CD133) in colorectal cancer


