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Expression of CEA and p16 in Colorectal Cancer

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ABSTRACT
Colorectal cancer is any cancer that affects the colon or rectum which is known to be the most common malignant cancer in the GIT. The aim of this case controlled retrospective study is to determine the expression of CEA and p16 in normal colorectal tissues, colonic polyps and colorectal carcinoma. IHC analysis of the expression of CEA and P16 was performed on a total of 65 formalin fixed paraffin-embedded tissue blocks including 15 normal colorectal tissues, 25 benign colonic polyps and 25 colorectal carcinoma were retrieved from pathology archives. Immunohistochemical analysis was carried out on the samples. The results obtained showed that nuclear CEA staining was expressed in which normal cases showed a positivity rate of 50%, colonic polyps showed a positivity rate of 90% and CRC showed a positivity rate of 100%. Staining of p16 was expressed in which normal cases showed a positivity rate of 20%, colonic polyps showed 70% and colorectal cancer showed 93%. A positive relationship between the degree of expression of CEA and p16 and the severity of the lesions in the progression of colorectal carcinoma was observed through this course of study. While these markers have been proven to be effective in predicting the progression of normal colorectal tissue to colorectal carcinoma based on their staining patterns, none of these markers can stand on its own to give fully definitive result and should be used in concordance with each other to compensate for their limitations and obtain relevant results.

Keywords: Carcinoembryonic antigen (CEA), p16, Colorectal cancer, Expression, Tumour

INTRODUCTION
The most prevalent malignant cancer in the GIT, accounting for 13% of all malignant tumors, is colorectal cancer (CRC), which is any cancer that affects the colon or rectum[1]. It ranks third for men and second for women in terms of cancer-related deaths. The death rate from colorectal cancer has been declining as a result of improvements in screening methods. Cancer of the colon can be benign or malignant[2]. The primary cause of mortality from gastrointestinal cancer and the

second most frequent cancer in both men and women worldwide are all colorectal cancers. Conversely, patients who receive a diagnosis earlier in life have a less aggressive condition[3]. The location, size, and presence or absence of metastases of the tumor all affects the colorectal cancer patient's clinical appearance. Clinical symptoms of colorectal cancer include changes in chronic bowel habits, abdominal pain, anorexia, and changes in bowel movements[4]. By implementing systematic interventions that include early detection, treatment services, prevention, and effective screening, colorectal cancer mortality rates could be decreased globally.

This research will aid in the early diagnosis and monitoring of already present malignancies through the expression of the chosen tumor markers; Carcinoembryonic antigen (CEA) and p16. The adoption of CEA as a tumor marker is due to the close association between colorectal cancer and CEA expression. The measurement of CEA levels in serum aids in the accurate and clinical detection of colorectal cancer. A prognostic indication for the condition of patients with colorectal cancer is an increase in CEA levels[5]. The development of colonic polyps into colorectal cancer from normal colorectal tissue is a rare opportunity for CRC prevention and early detection. For women, the CRC death rate has fallen since 1947; for men, it has only done so since 1980. Due to the inconsistent patterns of CRC risk variables, this discrepancy most likely reflects sex disparities in incidence trends. By sex, the trends over the last three decades are remarkably comparable. The 20th century witness a decline in mortality that is attributable to screening (53%), shifting trends in CRC risk factors (35%), and improvements in therapy (12%). The considerable decline in colorectal cancer-related mortality and morbidity rates, however, was largely the result of screening[6].

The selection of the immunohistochemical markers employed in this investigation was based on reports from the body of literature that the markers chosen were relevant to the development of colorectal cancer. A very good marker for the detection of colorectal cancer and its precancerous lesions is CEA and p16. High CEA levels are present in

around 70% of patients with CRC at the time of diagnosis[7]. In colorectal cancer, p16 expression has been examined, but the results have not been consistent[8]. According to the literature that is currently available, CEA and p16 are helpful markers for figuring out how the aforementioned cases are progressing because they show traditional tumor cell characteristics, such as cell proliferation and contact inhibition, which can predict and diagnose colorectal carcinoma as well as figure out the grade or expression of the cancer[9]. In order to observe the evolution of immunoreactivity from normal to colonic polyps to colorectal cancer and to ascertain the expression of particular genes, the study's findings would be helpful.

MATERIALS AND METHOD

Tissue Sample Selection

A total of 65 formalin fixed paraffin embedded tissue blocks comprising of 15 normal colorectal tissues, 25 colonic polyps tissues and 25 malignant invasive colorectal cancer were retrieved from the Pathological Archives of the Obafemi Awolowo University Teaching Hospital Complex Ile-Ife (OAUTHC).

Immunohistochemical Analysis

The expression of the biomarkers, CEA and p16, were demonstrated immunohistochemically using the Avidin-biotin immuno-peroxidase method. Sections on adhesive coated glass slides were deparaffinized in xylene and rehydrated using different gradients of ethanol. The sections were pretreated in a pressure cooker for antigen retrieval, using antigen retrieval buffer at 95°C for 30 minutes, 90°C for 10 seconds and 10°C for 10 minutes. Endogenous peroxidase activity was blocked by immersion in 3% hydrogen peroxidase solution for 5 minutes. Non-specific binding was blocked with the use of blocking buffer (horse non-immune serum) for 15 minutes. 200µl of diluted primary antibody (BioGenex mouse monoclonal primary antibodies) for *MSH2* and *MSH6* sequentially was added to slides and incubated at room temperature for 80 minutes. The slides were incubated with biotinylated rabbit anti-mouse secondary immunoglobulins for 15 minutes at room temperature. They were subsequently incubated with

avidin-biotin peroxidase complex. 3,3-diaminobenzidine was used as a chromogen. The sections were counter stained with hematoxylin.

Immuno-staining Assessment

Expression of CEA and p16 were determined through a semi-quantitative method. The immunoreactivity of these markers was determined by assessing the staining intensity and percentage of stained cells per field. The staining intensity was graded as mild, moderate and severe. The percentages of positive cells were graded as follows:
0.1%- 10% are stained = negative (-), grade 0.
10.1%- 39% are stained= positive (+), grade 1.
40. 0%-79% are stained= positive (++), grade 2.
80.0%-100% are stained = positive (+++), grade 3 (Ekundina *et al.*, 2021).

Analysis

Results were presented in figures and tables; pictures (micrographs) were also used where necessary. CEA and p16 staining was evaluated using regular light microscope at x100 and x400.

Photomicrography

The Stained sections were examined under a LEICA research microscope (LEICA DM750, Switzerland) interfaced with digital camera (LEICA ICC50). Digital photomicrographs of stained sections for the histomorphology and immunohistochemistry on the organs studied were taken at various magnifications and reported for Morphological changes.

RESULTS

Table 1 showed the staining intensity of p16 in the progression of Colorectal Cancer. The staining intensity assessed by semi quantitative method is shown to be increasing from the normal to the CRC. The Normal cases had a high negativity rate with 8 of 10 cases unreactive. The polyps showed moderate intensity with CRC

expressing moderately. The result also showed an increase in the positivity rate from the normal case to CRC.

Table 2 showed the percentile reaction of p16 in the progression of CRC. From the results obtained, the percentile negativity reduced from the normal to the CRC, while the percentile positivity increases from the normal to the CRC. Table 3 showed the staining intensity assessed by semi quantitative method. The staining intensity assessed by semi quantitative method is shown to be increasing from the normal to the CRC. The Normal cases had a negativity rate with 5 of 10 cases. The polyps showed moderate intensity with CRC expressing markedly. There was increase in the positivity rate from the normal case to CRC. Table 4 depicts the percentile rate of the CEA in the progression of CRC. The percentile negativity reduced from the normal to the CRC, while the percentile positivity increased from the normal to the CRC.

Figure 4 showed a graphical representation of the expression of the p16 in progression of colorectal cancer. The graph showed a progressive increase in staining intensity as seen during immunohistochemical staining with p16. Figure 5 showed a graphical representation of the expression of the CEA in progression of colorectal cancer. The graph showed a progressive increase in staining intensity seen during immunohistochemical staining with CEA. Figure 6 showed a graphical comparison between the staining reactions of the IHC markers. The p16 graph reveals its smooth progression from normal to CRC. CEA marker shows the same staining intensity across, the graph reveals that both markers are useful in colorectal diagnosis.

Table 1: The staining intensity of p16 in the progression of Colorectal Cancer

Group (p16)	Total cases	-	+	++	+++	Mean Percentage Reactivity
Normal/Control	10	8	2	0	0	15%
Polyps	20	6	6	8	0	56%
CRC	30	2	6	9	11	81%

Table 2: The percentile reaction of p16 in the progression of CRC

Groups(MSH)	N	NEGATIVE (n %)	POSITIVE (n %)
Normal	10	8 (80%)	3 (20%)
CIN1	20	6 (30%)	14 (70%)
CIN2&CIN3	30	2 (7%)	28 (93%)

Table 3: The staining intensity of CEA in the progression of CRC

Group(CEA)	Total cases	-	+	++	+++	Mean Percentage Reactivity
Normal	10	5	5	0	0	25%
Polyps	20	2	5	13	0	65%
CRC	30	0	4	10	16	86%

Table 4: The percentile reaction of CEA in the progression of CRC

GROUP (CEA)	N	NEGATIVE (n %)	POSITIVE (n %)
Normal	10	5 (50%)	5 (50%)
Polyps	20	2 (10%)	18 (90%)
CRC	30	0 (0%)	30 (100%)

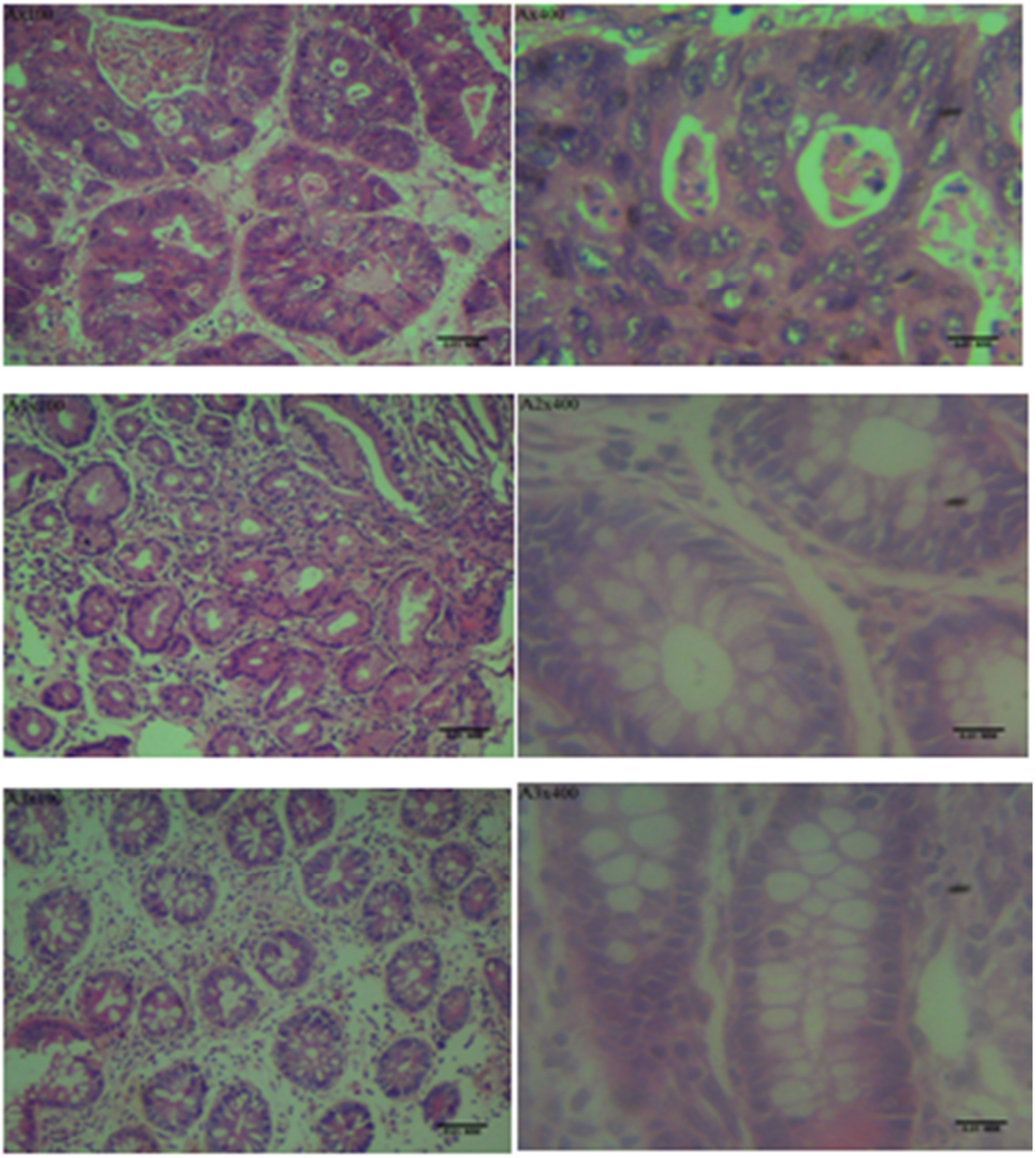

Figure 1: Micrograph of the normal colon stained with H&E: (A3) (NORMAL) well- formed glands with spaced lumen, the nucleus stained purple and cytoplasm stained pink. (A2) (polyps) the glands are partially occluded and mild dysplasia. The nucleus stained purple and cytoplasm stained pink. (A) (CRC) shows the lack of lumen of the gland, full occlusion and severe dysplasia, hyperchromasia is seen in staining.

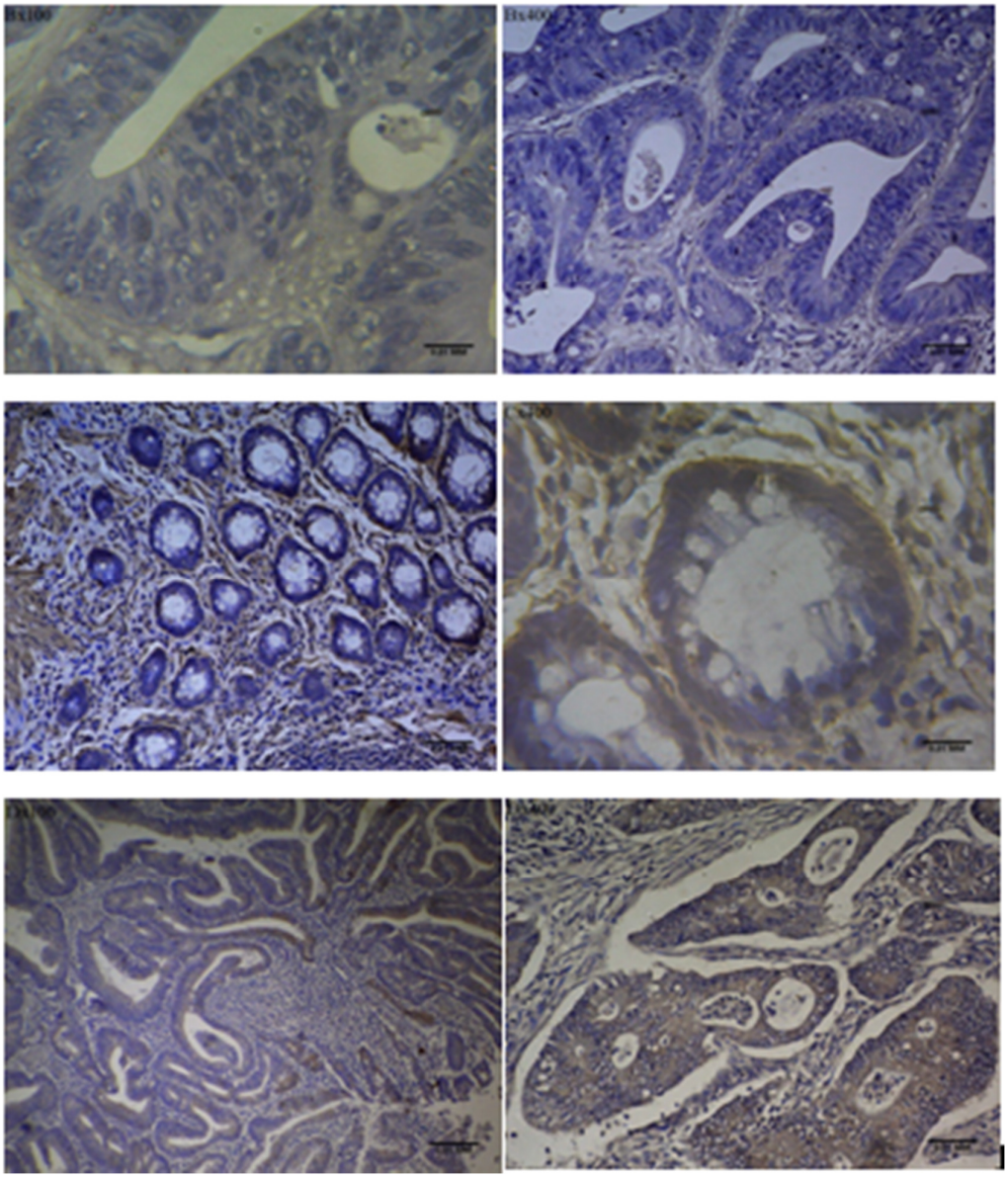

Figure 2: micrographs of the colorectal sections stained with P16 illustrating: (B) (NORMAL P5 p16 x100 & 400 respectively) shows no staining reaction (C) (polyps P16 x100 & 400 respectively) shows a mild staining intensity. (D) (CRC P16 x100 & x 400 respectively) shows a moderate staining intensity and occlusion of ducts.

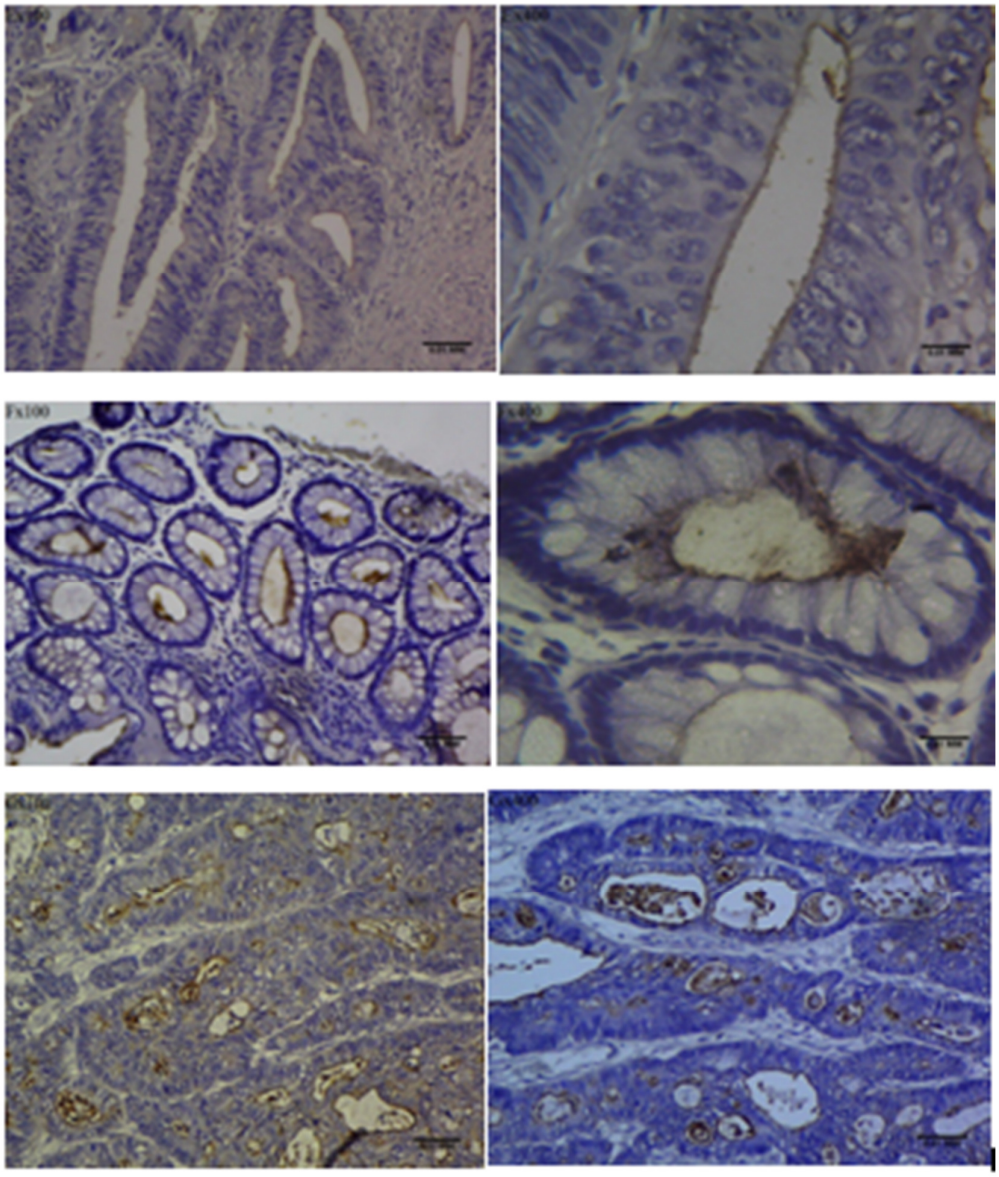

Figure 3: micrographs of the colorectal sections stained with CEA illustrating: (E) (NORMAL CEA x100 & 400 respectively) shows mild staining reaction (F) (polyps CEA x100 & 400 respectively) shows a moderate staining intensity. (G) (CRC CEA x100 & x 400 respectively) shows a severe staining intensity.

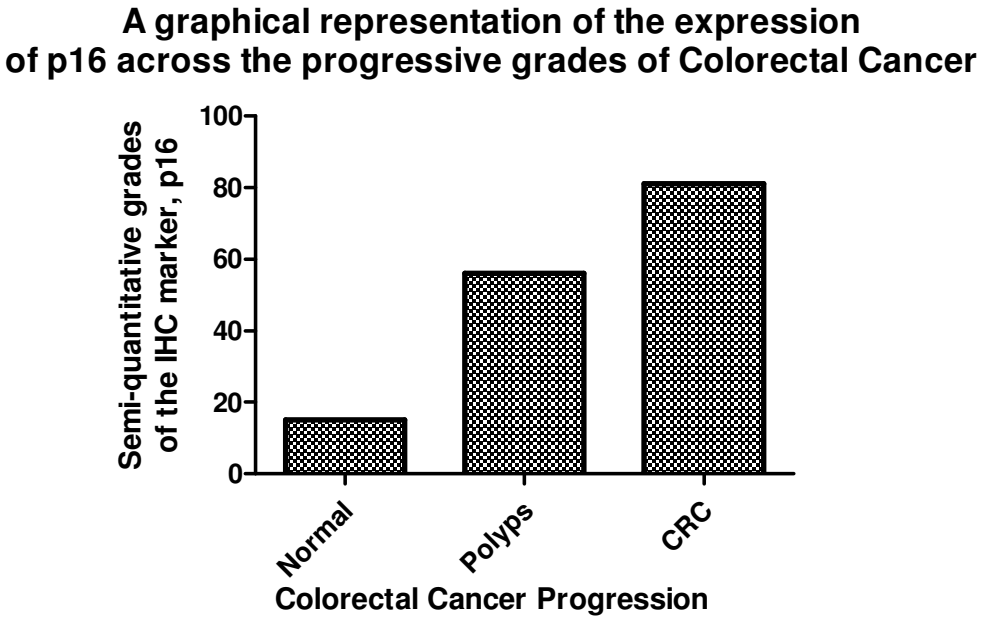

Figure 4: A graphical representation of the expression of the p16 in progression of colorectal cancer.

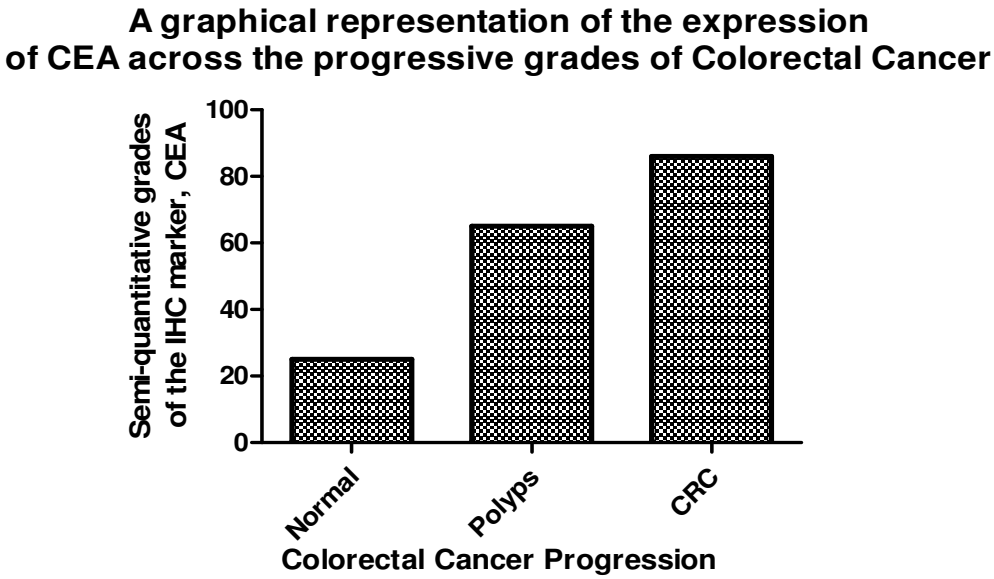

Figure 5: A graphical representation of the expression of the CEA in progression of colorectal cancer.

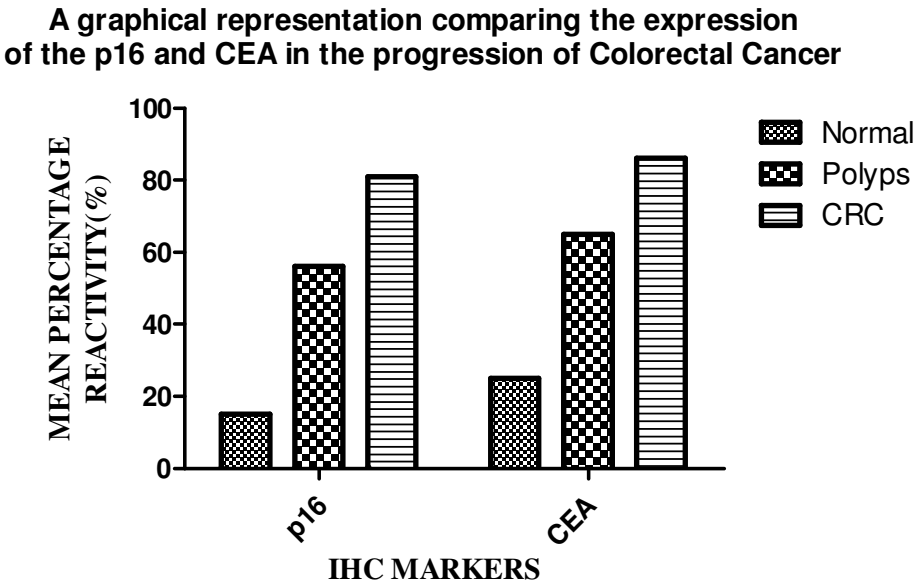

Figure 6: The above graph compares the staining reaction of the IHC markers.

DISCUSSION

The second most common disease in women and the third most common in males, colorectal cancer accounts for around 10% of all cancer diagnoses and cancer-related deaths worldwide each year[9]. With the purpose of determining their predictive value in the development of colorectal cancer, the expression of CEA and p16 Immunohistochemical (IHC) markers was evaluated. The ability to forecast the progression of a colorectal lesion to the malignant state depends on the observation and identification of benign or premalignant colorectal lesions as they evolve to a malignant condition. The aim of this case controlled retrospective study was to determine the expression of CEA and p16 in normal colorectal tissues, colonic polyps and colorectal carcinoma and to determine the degree of expression of these biomarkers.

The protein p16, also known as $p16^{INK4a}$, cyclin-dependent kinase inhibitor 2A, *CDKN2A*, and multiple tumor suppressor 1, is a tumor suppressor that inhibits cell division by delaying the transition from the G1 to the S phases of the cell cycle[10]. The *CDKN2A* gene

produces its protein. A deletion in this gene can lead to insufficient or non-functional p16, speeding the cell cycle and resulting in a variety of cancers. A deletion is the removal of a portion of the DNA sequence during replication. The histological diagnosis accuracy of grade III cervical intraepithelial neoplasia can be increased by using p16 as a biomarker (CIN). Furthermore, p16 is linked to the protection of esophageal, cervical, vulvar, and oropharyngeal cancers as well as melanoma[11]. The name p16 comes from its molecular weight, and its alternate name, p16INK4a, relates to its function as a *CDK4* inhibitor. The interplay of numerous transcription factors, as well as several proteins involved in epigenetic modification through methylation and repression of the promoter region, is necessary for the complex regulation of p16[12].

Inactivation of $p16^{INK4a}$ occurs in a sizeable number of colorectal tumors (18-53%), with denovo methylation of its 5o-promoter-associated *CpG* Island being the most frequent mechanism. Studies have demonstrated that $p16^{INK4a}$ is a tumor suppressor as well as a cell cycle regulator. Although Kamoshida *et al.*[13] claim a connection between $p16^{INK4a}$ methylation and poorer survival in colorectal cancer has been demonstrated, this current investigation reveals the p16 predictive potential. This research confirmed Testa *et al.*[14] findings that there is a significant difference between immunohistochemical staining in colonic polyps and the invasive cancer due to the over expression of the mutated forms of the p16 found in oncogenic cells. In this study, p16 was seen to progressively increase across the malignant and premalignant stages of colorectal cancer.

The fact that the p16 is a cell cycle checkpoint for defects in cell proliferation, as explained by Al-Ghafri *et al.*[15], and that immunohistochemistry looks for the mutated form of the p16 means that it may not be found in the normal colon. The normal colon is almost devoid of a staining reaction in the reaction. Yet, as traces of the p16 mutation start to occur, the benign stage of colorectal cancer exhibits a moderate staining intensity. This mild staining intensity becomes noticeable in the CRC due to an increase in p16 mutation in

the cells, depending on the length of the benign state in the visibility of the reaction. Since there is a large difference between benign and malignant lesions, p16 has strong diagnostic value. This is consistent with earlier studies that claim p16 has poor prognostic value because of the marker's frequent expression[15-16].

Previous clinical study have also revealed that p16 was inactivated by point mutation, promoter hypermethylation, or homozygous deletion which was commonly found in many human cancers[17]. The genetic variation of *p16* gene was commonly found in patients with cancer, and p16 had a crucial role in the process of cell growth. For instance, the status of *p16* gene promoter methylation was commonly studied and significantly associated with the development of bladder cancer, lung cancer, brain cancer, and esophagus cancer[18-19]. At the same time, the study of Chen *et al.*[20] has suggested that promoter hypermethylation of the *p16* gene might be significantly associated with the clinicopathologic features of CRC. However, an accurate expression level of *p16* gene was not detected in these studies despite the significant impact of p16 promoter hypermethylation on p16 protein expression[20].

In individuals with CRC, carcinoembryonic antigen (CEA) is one of the most prevalent and practical markers. It has been employed in the diagnosis of cancer, the assessment of therapeutic response, prognostication, or the detection of recurrence[21]. Although CEA is not a disease-specific marker, it has been proposed that a rise in CEA levels from preoperative to postoperative levels is a sign of a higher recurrence rate. Several research have looked into CEA's potential as a prognostic indicator in CRC[22]. CEA is a glycoprotein expressed on colonic epithelial cells and secreted into the bloodstream, leading to an increase in serum level[23]. The biological activity of malignancies is assessed by CEA, which has a high sensitivity for detecting recurrence. Hence, it was recommended by the American Society of Clinical Oncology and the European Society for Medical Oncology as a prognostic biomarker during routine follow-up for CRC after surgical resection[24].

CEA, a heavily glycosylated macromolecule with a complicated structure found in colon cancer does not appear to be present in the healthy adult colon[25]. In this study, the CEA and p16 behave alike as they progressively increase across the progression of the colorectal cancer. CEA in this present study is seen to be mildly present in the normal; however this cannot serve as a tool for early detection of cancer because expression of CEA in the normal cell can be caused by a wide range of factors that are not necessarily oncogenic[26]. The mild reaction of the normal cells progressively moves to the moderate reaction in the polyps, which is of diagnostic importance in agreement with Testa *et al*[14]. The CEA expression in the benign lesion can be used to predict the occurrence of the invasive cancer; the CEA has a diagnostic possibility for colorectal cancer. Kim *et al.*[27] also agrees with the over expression in the malignant lesions which is due to the cell adhesion properties of the CEA, this hereby emphasizes the known fact that oncogenic cells are always closely packed together losing their well defined cell junctions. This study shows that CEA produces a significant difference between benign and malignant lesions so can be a good predicting factor. According to Tiernan *et al.*[28], when compared to other markers, differential CEA expression among normal/tumour pairs was significantly higher, indicating that CEA is the most reliable marker for distinguishing between normal and tumor tissue.

CONCLUSION

From the statistical data gathered from this retrospective study, it has been shown that the p16 and CEA are expressed in premalignant and malignant lesions with moderate and severe intensity respectively, and their expressions are directly related to the increasing grades of colorectal cancer. The data reveals that the IHC markers are good diagnostic markers, however no marker should be independently used, and markers should be used in pairs for optimal results.

Conflict of Interest

The authors declare no conflict of interest.

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