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Immunohistochemical study of the expression of OCT4 and SOX2 in non-neoplastic, and neoplastic colorectal tumors.

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Abstract: Background: Colorectal cancer (CRC) is one of the commonest malignancies worldwide and it is considered the second cause of cancer deaths in the western countries. OCT4 gene, found the earliest and a very important key gene in embryonic stem cell self-renewal and differentiation potential, together with SOX2 and Nanog, also as markers of stem cells. SOX2 expression has been linked with a stem cell state in many human tumors such as, ovarian, cervical, head and neck squamous cell carcinoma, and breast carcinoma, but so far this has not been studied in CRC. SOX2 expression was associated with poorly differentiated tumors (high grade) in different cancers. **Aim:** Compare between expression of OCT4 and SOX2 with non-neoplastic and neoplastic colorectal lesions. Correlate their expression with clinicopathological parameters. **Results**: there was a significant relation between sox2 expression with development of neoplasia and malignancy in colorectal lesions. A significant relation was found also between Sox2 expression and tumor size and stage. OCT4 expression was associated with high tumor grade, size and stage of malignant tumors. OCT4 expression on the other hand, has no significant relation with development of malignancy. **Conclusion**: SOX2 is a reliable marker for prediction of progression of malignancy in colorectal lesions unlike OCT4 which can't be used alone in predicting development of malignancy. The combination of the two markers is useful in predicting progression of colorectal carcinoma.
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Key words: OCT4, SOX2, colorectal cancer

1. Introduction

Colorectal cancer (CRC) is one of the commonest malignancies worldwide and in the western world it is considered the second cause of cancer deaths. high mortality rate seen in CRC is often due to late diagnosis in advanced stages. Nowadays, most patients receive more or less similar stage treatment regimens, although not all get benefit from it. In future treatment strategy of CRC patients, personalized therapy will require great importance, but this will also make higher demands for molecular subclassification of colon cancer **(Siegel *et al.*, 2014).**

One recent research revealed that, a one small subgroup of tumor cells have characters related to stem cells. So, they are called cancer stem cells (CSCs). CSCs have the cabability of self-regeneration and multilineage differentiation. Those features can cause both start of new tumors and tumor growth **(O'Connor *et al.*, 2014)**

CSC-like normal stem cells have the ability to self-renewal and multilineage differentiation, with unlimited proliferation and migration ability. But, there is no regulation mechanism of CSC's self-renewal and differentiation, which makes the proliferation get beyond control and does not have the ability to differentiate into mature cells **(Chen *et al.*, 2017)**

OCT4 gene, found the earliest and a very important key gene in embryonic stem cell self-renewal and differentiation potential, together with SOX2 and Nanog, also as markers of stem cells. In other studies, OCT4 gene was expressed in both embryonic and germ cell tumors, and higher expression of OCT4 was also found in tumor tissues and cells of the reproductive system, skin squamous cell carcinoma, hepatocellular carcinoma, gastric cancer, esophageal cancer and breast cancer **(Huang *et al.*, 2010)**

Abnormal expression of OCT4 gene may have a role in the molecular mechanism of tumor cell formation, and it is also one of the important causes of stem cell carcinogenesis. Many researches have found that OCT4 have the ability to maintain embryonic stem cells and adult stem cells pluripotency and self-renewal. The high expression of OCT4 gene is related to the maintenance of the cell, and its down-regulated expression is related to the differentiation (**Rodriguez *et al.*, 2007).**

OCT4 silencing can make murine and human embryonic stem cells differentiated into trophoblast cells. But, few papers have proposed the opposite

conclusion. Therefore, the OCT4 gene requires more studies in the maintenance of adult stem cell multi potential and self-renewal **(Zhang *et al*., 2017).**

SOX2 (Sex-determining region Y (SRY)-Box2) is considered one of the members of the SOX family of transcription factors. This family is responsible for coordinating different functions like maintaining stem cell characteristics and differentiation ability. In particular, SOX2 has an important role in the regulation of stem cell fate during embryonic development. Its expression levels also need tight regulation to make sure that normal embryonic development has done. SOX2 depletion by RNA interference enhances embryonic stem cell differentiation into different cell types **(Herreros *et al*., 2013).**

SOX2 expression has been related to stem cell state in many human tumors such as, ovarian, pancreatic, squamous cell carcinoma of head and neck, and carcinoma of breast, but so far this has not been studied well in colon cancer. SOX2 expression was related to higher grade tumor (poorly differentiated) in different cancers **(Lundberg *et al*., 2014; Pham *et al*., 2013).**

The role of OCT4 and SOX2 in colon cancer has been confirmed and as tumor markers in diagnosis and prognosis of colon tumors. These results are also confirming the role of the theory of cancer stem cell in cancer **(Amini *et al*., 2010).**

Aim of the work:

Study the relation between OCT4 and SOX2 expression with development of malignancy in colorectal lesions, and correlate their expression with clinicopathological parameters of the tumor.

2. Patients and methods

Patients and specimens.

CRC tissues, various types of adenomas and benign polyp tissues were used in this study. They represent steps in the evolution of CRC. All tissues used in this study, either primary, or paraffin-embedded sections were obtained from department of pathology, Tanta University and from private laboratories. None of the patients received rather radiotherapy or chemotherapy as a preoperative treatment.

Primary specimens of CRC and non-tumor specimens were obtained from 20 patients with CRC, who underwent radical tumor resection. Benign polyps and adenomas were obtained from 5 and 20 patients respectively, who were undergoing endoscopic removal.

IHC methodology.

Formalin-fixed paraffin-embedded sections (4-µm) were fixed on to APES-coated glass slides (Chenglin, Shanghai, China). Slides were put in xylene for dewaxing twice for 10 min. Then rehydration was done through emerging in graded ethanol. Antigen retrieval step was made in 0.01 mol/l citrate buffer (pH 6.0) by 10 min boiling. Endogenous peroxidase activity was decreased with 3% hydrogen peroxide for also 10 min. After washing with phosphate-buffered saline (PBS), slides were blocked against background staining with 5% BSA for 30 min at 37˚C. Sections were incubated overnight with mouse primary monoclonal antibody for human Oct-4 ((CA 95677) (Cell marque, CA, USA)) and monoclonal mouse anti-SOX2, Diagnostic BioSystem, Clone ≠ NRG5.6) at 4˚C, in a humid chamber. After three times washing with PBS, sections were incubated with the secondary antibody (goat anti-mouse IgG peroxidase in a dilution of 1:300; catalog no. 32230; Zymed, San Diego, CA, USA) for 30 min. After three times washing again in PBS, 3,3'-diaminobenzidine (as chromogen) was put. Slides were counter-stained for 1 min with hematoxylin. For a negative control, sections were not incubated with the primary antibody.

Scoring of OCT4 and SOX2

Two blinded investigators separately evaluated the results of immunostaining. According to Zhou et al's 2013 reported method, OCT4 positive cells appeared as nuclear stain with brown yellow granules, and the final assessment was by adding a percentage score for the total number of the same type of cells and the score of color intensity of positive cells. According to the percentage of positive cells score: No positive cell count as 0 points, positive cells from 1-25% as 1 point, from 26-50% as 2 points, from 51-75% as 3 points, > 75% as 4 points. According to the intensity of color: no color as 0 points, weak staining as 1 point, moderate staining as 2 points, intense staining as 3 points.

According to the sum of the two indexes, the results were classified into 4 grades:

0 points as (-), 1-4 points as weak positivity (+), 5-8 points as moderate positivity (+ +), 9-12 points as strong positivity (+ + +). From + to +++ was counted as a positive expression **(Zhou *et al*., 2013).**

Immunopositivity of SOX2 was detected by dark brown staining of nucleus of cells. The sections were counted and analyzed. Five high‐power fields (×400) were randomly selected in each slide, and a total of 1000 cells were counted by two separate investigators. If <5% of the nuclei showed positivity, it was considered negative. If the percentage ranged between 5% and 25%, 26% and 50%, and >50%, scores of 1, 2, and 3 were recorded, respectively (**Alokenath, 2017).**

3. Results

This study was carried on 45 specimens of colorectal tissues, which were divided into (5

specimens of non-neoplastic polyps, 20 specimens of adenomas and 20 specimens of colorectal carcinomas. The age of patients ranged from 30 to 78 with a mean of 57.17 ± 12.12. Thirty-one patients were male and fourteen were females.

Cases of colorectal carcinomas include various stages (I, II, III, IV) and various grades (II, III). One case was of stage I, eight cases were of stage II, nine cases were of stage III and two cases were of stage IV. Regarding tumor grade, twelve cases were of grade II and eight cases were of grade III.

SOX2 results (table 1):

SOX2 was detected as brown nuclear staining of the tumor cells. Positivity of SOX2 was significantly increased with the increase of tumor size (P=0.001). SOX2 expression was significantly associated with adenocarcinoma. Most of cases of adenocarcinoma (15 out of 20) showed SOX2 expression (fig 1,2). While, only 4 out of 20 cases of adenomas express SOX2 (fig 3) and 2 out of 5 non-neoplastic polyps were positive to SOX2 (fig 4). This relation was statistically significant (p=0.005).

Studying the relation of SOX2 results with stage of the tumor also revealed significant relation. All the cases of stage I was negative to SOX2. While all cases of stage IV were strongly positive to SOX2.

On the other hand, no significant association was found between SOX2 expression and tumor grade, site, age or gender of patients.

Table 1: SOX2 with patients and disease criteria:

Variables	SOX 2				Test of sig	P value
	0	I	II	III		
Age (mean ±SD)	57.37 ± 14.43	57.88 ± 7.32	54.37 ± 4.03	60.00 ± 18.54	K=2.19	0.53
Size (mean ± SD) Malignant only	3.20 ± 0.27	3.83 ± 0.25	4.40 ± 0.22	5.50 ± 0.57	17.16	0.001
Diagnosis Adenoma (no=20) Polyp (no=5) Adenocarcinoma (no=20)	 16 (80.0) 3 (60.0) 5 (25.0)	 1 (5.0) 2 (40.0) 6 (30.0)	 3 (15.0) 0 (0.0) 5 (25.0)	 0 (0.0) 0 (0.0) 4 (20.0)	 15.51	 0.005
Stage (of adenocarcinoma) I (no=1) II (no=8 III (no=9) IV (no=2)	 1 (100.0) 4 (50.0) 0 (0.0) 0 (0.0)	 0 (0.0) 3 (37.5) 3 (33.3) 0 (0.0)	 0 (0.0) 1 (12.5) 4 (44.4) 0 (0.0)	 0 (0.0) 0 (0.0) 2 (22.2) 2 (100.0)	 14.75	 0.01
Grade (of adenocarcinoma) II (no=12) III (no=8)	 4 (33.3) 1 (12.5)	 3 (25.0) 3 (37.5)	 2 (16.7) 3 (37.5)	 3 (25.0) 1 (12.5)	 FE=2.26	 0.60
Metastatic LN (of adenocarcinoma) Negative (no=12) Positive (no=8)	 5 (41.7) 0 (0.0)	 3 (25.0) 3 (37.5)	 2 (16.7) 3 (37.5)	 2 (16.7) 2 (25.0)	 FE=4.67	 0.23

OCT4 results (table 2):

OCT4 was detected by brown nuclear staining of tumor cells. Increasing positivity of OCT4 was associated with increase of size of malignant tumors (P=0.001). All cases of stage I were negative to OCT4. All the cases of stage IV were strongly positive to OCT4. All cases of stage III were also positive to OCT4 expression. This relation was statistically highly significant (P=0.001).

Thirteen cases out of 20 adenocarcinomas were positive to OCT4 while only 7 were negative. (Fig 5,6) Only 6 out of 20 cases of adenomas were positive to OCT4 (Fig 7) while the majority was negative (14 cases). Only one case of non-neoplastic polyp was positive to OCT4 (Fig 8). In spite of that, this association was statistically non-significant (P=0.24).

OCT4 expression was associated with increase in tumor grade. All of adenocarcinoma grade III were positive to OCT4, five out of 8 cases were associated with high OCT4 expression (+2,+3). While seven out of twelve cases of adenocarcinoma were negative to OCT4, the remaining of positive grade II cases showed variable positivity to OCT4. This relation was statistically significant.

Concerning the age and gender of malignant tumor no statistical significant relation was found between OCT4 expression on one hand and patient's age, gender, or the site of malignant tumors.

Table 2: OCT 4 with patients and disease criteria:

Variables	OCT4				Test of sig	P value
	0	I	II	III		
Age (mean ±SD)	57.24 ± 14.11	55.54 ± 6.93	60.16 ± 6.70	56.66 ± 21.19	K=0.65	0.88
Size (mean ± SD) Malignant only	3.28 ± 0.26	4.16 ± 0.25	4.50 ± 0.40	5.66 ± 0.57	F=34.90	<0.001
Diagnosis Adenoma (no=20) Polyp (no=5) Adenocarcinoma (no=20)	 14 (70.0) 4 (80.0) 7 (35.0)	 4 (20.0) 1 (20.0) 6 (30.0)	 2 (10.0) 0 (0.0) 4 (20.0)	 0 (0.0) 0 (0.0) 3 (15.0)	 7.23	 0.24
Stage (of adenocarcinoma) I (no=1) II (no=8 III (no=9) IV (no=2)	 1 (100.0) 6 (75.0) 0 (0.0) 0 (0.0)	 0 (0.0) 2 (25.0) 4 (44.4) 0 (0.0)	 0 (0.0) 0 (0.0) 4 (44.4) 0 (0.0)	 0 (0.0) 0 (0.0) 1 (12.5) 2 (100.0)	 19.78	 <0.001
Grade (of adenocarcinoma) II (no=12) III (no=8)	 7 (58.3) 0 (0.0)	 3 (25.0) 3 (37.5)	 1 (8.3) 3 (37.5)	 1 (8.3) 2 (25.0.)	 8.06	 0.02
Metastatic LN (of adenocarcinoma) Negative (no=12) Positive (no=8)	 5 (41.7) 2 (25.0)	 2 (16.7) 4 (50.0)	 3 (25.0) 1 (12.5)	 2 (16.7) 1 (12.5)	 2.55	 0.58

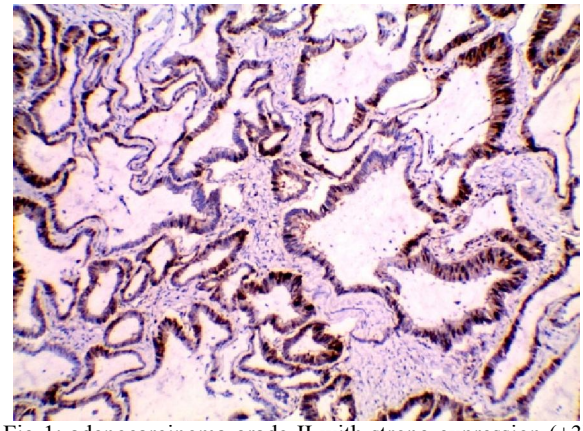

Fig 1: adenocarcinoma grade II with strong expression (+3) of SOX2 (Original magnification X 200)4.

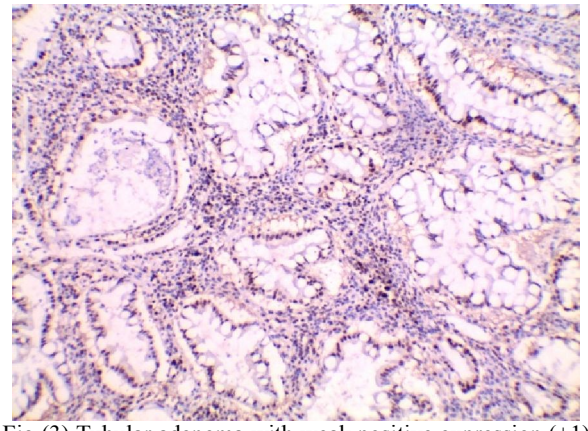

Fig (3) Tubular adenoma with weak positive expression (+1) of SOX2 (Original magnification X200)

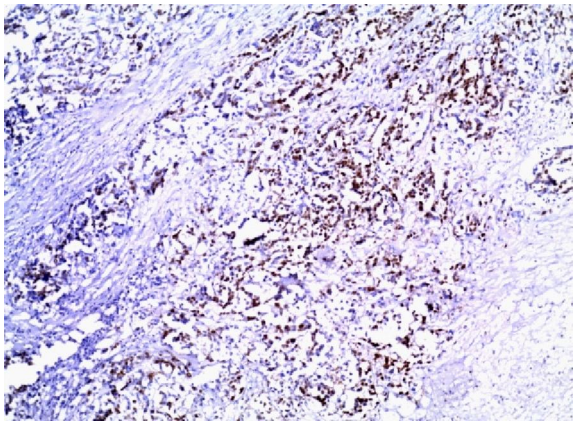

Fig 2: adenocarcinoma grade III with moderate expression (+2) of SOX2 (Original magnification X100)

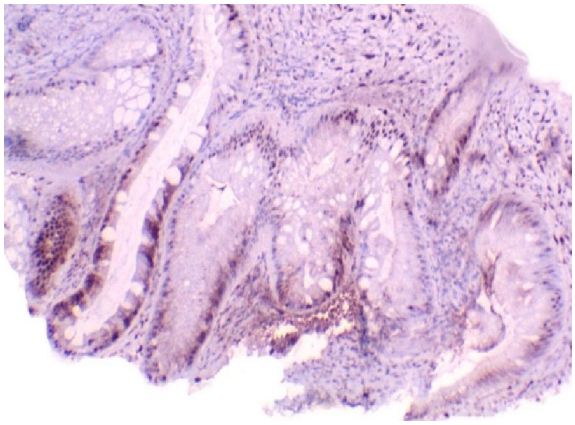

Fig (4): Hyperplastic polyp with weak expression (+1) of SOX2 (Original magnification X400)

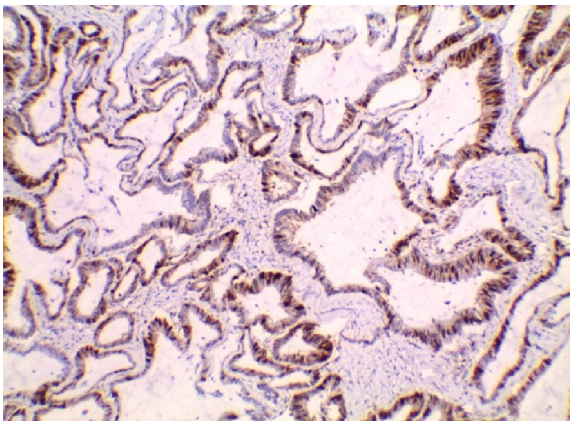

Fig 5: adenocarcinoma grade II with strong expression (+3) of OCT4 (Original magnification X200)

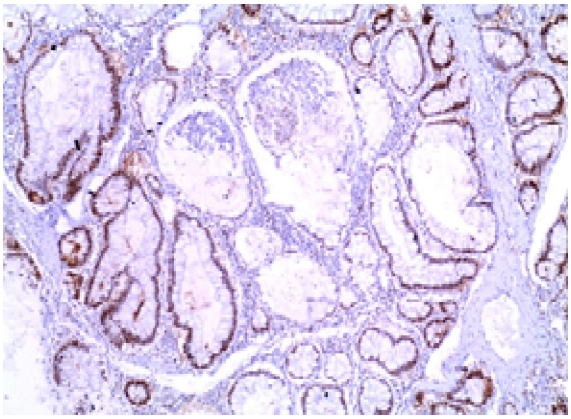

Fig 7: tubular adenoma with weak expression (+1) of OCT4 (Original magnification X200)

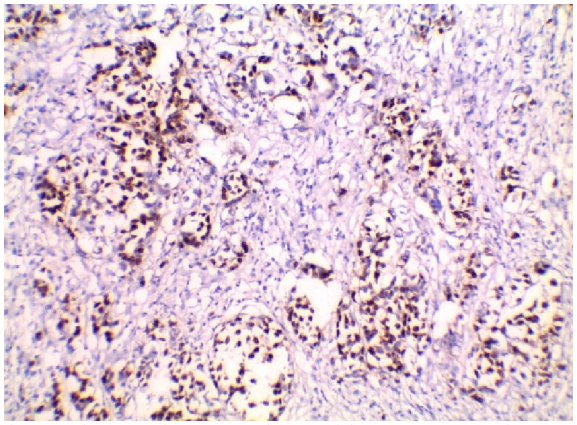

Fig 6: Adenocarcinoma grade III with strong expression (+3) of OCT4 (Original magnification X200).

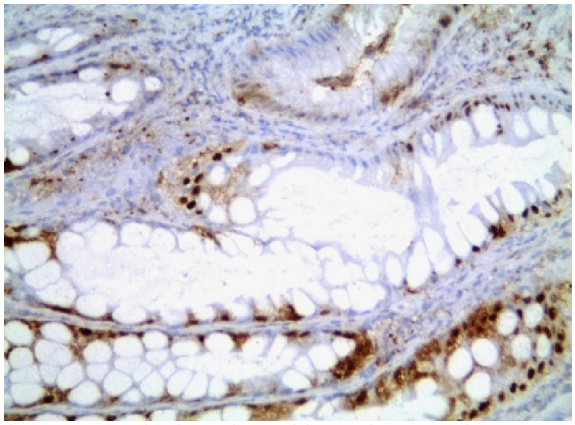

Fig 8: Hyperplastic polyp with weak expression (+1) of OCT4 (Original magnification X200)

4. Discussion

This study was carried on 45 specimens of colorectal tissues, which were divided into (5 specimens of non-neoplastic polyps, 20 specimens of adenomas and 20 specimens of colorectal carcinomas. The age of patients ranged from 30 to 78 with a mean of 57.17 ± 12.12. Thirty-one patients were male and fourteen were females.

This finding was higher than that obtained by **Wong *et al.* (2017)**, who found that colorectal neoplasia was three times more likely to be found in a 45- to 49-year-old man compared with a 40- to 44-year-old woman. This study found that men, advancing age, can be used to risk stratify for neoplasia development.

In the present work, as regards the grade and the stage of colorectal carcinoma cases, the most common grade was grade II (26.7%) followed by grade III (17.8%). Stage II A was the commonest stage (11.1%) followed by stage II (25%), then stage III A (4.4%) then stage II B and stage III C (6.7%) lastly stage III B and stage IV (4.4%). The high percentage of stage I might be due to early diagnosis after annoying symptoms (for example: abnormal uterine bleeding) which make patient seeking for consultation.

This finding was in coincidence with the results stated by **Rakislova *et al.* (2017)** who find that the most common grade in their study was grade II (26.7%) and but this study found that the most common stage was stage III (37.2%).

The present work aimed to study the relationship between SOX2 a OCT-4 immunohistochemical expression and clinicopathologic data of colorecral lesions.

OCT4, SOX2 are considered the genes that play a major role in regulating pluripotency. They are transcription factors which maintain self-regulation for their own expression through binding to their own promoter region, together with the expression of all three documented in a range of malignancies (**Munro *et al.*, 2017).**

Sox2 (Sry-box2) is important for a multiple of stem cells and it is also expressed in colorectal cancer (CRC). On the other hand, the mechanism by which Sox2 promotes CRC progression is still unclear. In the present study, we found that high Sox2 expression is

significantly correlated with bad prognosis (**Zheng, *et al*., 2017).**

In the present study, Sox2 expression in colonic adenoma cases was 20%,40% of colorectal polyp cases, and 75% of colorectal adenocarcinoma cases. This finding was higher than that reported by who found that (**Zheng *et al*., 2017).** The percentage of Sox2 cells in nonmalignant colorectal tissues was 37.78% and 80.85% of colorectal carcinoma cases.

According to stage of colorectal carcinoma, Sox2 was negative in all stage I cases, 50% of stage II cases, all of stage III cases, and all stage IV cases. According to grade of colorectal carcinoma, OCT4 was positive in 66.7 % of grade II cases and positive in 87.5 of grade III cases. According to metastasis to lymph node, OCT4 was positive 58.3% of colorectal carcinoma cases without metastasis to lymph nodes and it was positive in all of colorectal carcinoma cases with metastasis to lymph nodes.

Zheng, *et al*. (2017) found that Sox2 is pivotal in the regulation motility and progression of colon cancer. Sox2 expression was more in the early stages of the disease, suggesting that up-regulation of the gene may be the early event in the genesis of colorectal cancer. Their data showed that higher expression of Sox2 in tumors graded as Duke's B more than that in Duke's C or Duke's D tumors.

In the present study, OCT-4 expression in colonic adenoma cases was 30%, 20% of colorectal polyp cases, and 65% of colorectal adenocarcinoma cases. This finding was higher than that reported by **Huanzhou *et al.* (2015**) who found that low Oct4 expression were detected in normal colorectal tissues, however, the number was significantly higher in benign polyp tissues. It was also significantly increased in CRC tissues in comparison with benign polyp. The percentage of Oct-4+ cells in the polyps, adenomas and malignant colorectal tissues was 1.40±0.78, 2.91±1.57 and 11.37±6.32% respectively, with a significant difference was detected in the three groups.

Dai *et al.* (2013) found that Oct-4 increased in a progressive manner, from non-tumor to benign polyp and from benign polyp to adenoma and malignant tumor tissues.

According to stage of colorectal carcinoma, OCT4 was negative in all stage I cases, 25% of stage II cases, 88.9% of stage III cases, and all stage IV cases. According to grade of colorectal carcinoma, OCT4 was positive in 41.7 % of grade II cases and positive in all grade III cases. According to metastasis to lymph node, OCT4 was positive 58.3% of colorectal carcinoma cases without metastasis to lymph nodes and it was positive in 75% of colorectal carcinoma cases with metastasis to lymph nodes.

Hu *et al*. (2017) revealed expression of another cancer stem cell markers ABCG2 and OCT-4 in colon cancer that capable to predicts recurrence and poor clinical fate. **Dai *et al.* (2013)** did not found a statistical significant correlations between OCT-4 expression with either TNM stage (p=0.143), the differentiation or grade of tumor (p=0.055), or lymphovascular invasion (p=0.063).

However, **Papagiorgis *et al*. (2012)** found no significant association between OCT-4 expressions with the clinicopathological data of RCC. OCT-4 may act as an ON and OFF switch in CSCs. Moreover, although OCT-4 expression is usually stable, OCT-4 regulation is a complex process and mainly depends on the surrounding microenvironment.

OCT-4 overexpression together with the decrease of SOX2 expression is strongly associated with bad clinical outcome in cervical cancer (**Kim *et al*., 2015). These** results are similar to our results.

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