Original Article

Comparison of gene expression of SOX2 and OCT4 in normal tissue, polyps, and colon adenocarcinoma using immunohistochemical staining

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Abstract

Background: Cancer stem cells have been isolated and characterized in all common cancers. SOX2 and OCT4 are important genes to enhance the self-renewal ability as activate stem cells and inhibit the genes that start differentiation and thus maintain the self-renewal ability of stem cells. Also, the aim of this study is "Comparison of gene expression of SOX2 and OCT4 in normal tissue, polyps, and colon adenocarcinoma using immunohistochemical staining."

Materials and Methods: This cross-sectional study conducted on 20 patients so that for each patient, a sample of healthy tissue, dysplastic polyp tissue, and colon adenocarcinoma were provided as microscopic sections and staining on each tissue was performed through immunohistochemistry method by markers OCT4 and SOX2. The collected data were interred into SPSS version 18.0, (SPSS Inc., Chicago, IL, USA) software and the level of significance were considered as <0.05.

Results: The study sample consisted of 20 patients including 11 men (55%) and 9 women (45%) with a mean age of 55.6 ± 9.88 years. There was no association between Oct4 and colorectal cancer (CRC) patients ($P > 0.05$), but there was a significant correlation between Sox2 expression and CRC ($P < 0.05$). Patients in many aspects such as race, type of polyp, presence of lymph node, grade and intensity of Sox2 in different types of patients' tissues ($P < 0.05$).

Conclusion: Regarding our findings, the expression of Sox2 would be a liable marker for evaluating of cancer progression and could be a treatment target of CRC cells.

Key Words: Colon adenocarcinoma, colorectal cancer, dysplastic polyp, immunohistochemical, OCT4, SOX2

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INTRODUCTION

Colon cancer after lung cancer is the most important cause of cancer-related deaths in the world.[1] According to the new theory of stem cell origin of cancer, most tumors originate from cancer stem cells. According to this theory, nowadays the factors that cause inhibiting the process of the differentiation and uncontrolled proliferation of tissue stem cells are the most important factors in the carcinogenesis process.[2,3]

SOX2 (a member of the Sox [SRY-Related HMG box] gene family) and OCT4 (a member of the

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POU family) are important genes to enhance the self-renewal ability as activate stem cells and inhibit the genes that start differentiation and thus maintain self-renewal ability of stem cells.[4,5] So these markers have a nuclear pattern in immunohistochemistry (IHC). The level of Oct4 and Sox2 mRNA in peripheral blood of patients with metastatic colorectal cancer (CRC) was found to be higher than in healthy controls by quantitative real-time-polymerase chain reaction (RT-PCR).[6] Also, in rectal cancer, higher mRNA levels of Oct4 and Sox2 in residual tumors after preoperative chemoradiotherapy were associated with higher rates of metastatic relapse.[7]

Cancer stem cells with metastatic potential represent poor resistance to treatment and prognosis. Recurrence of the tumor is one of the major causes of morbidity and mortality in patients with rectal cancer undergoing chemoradiotherapy. Therefore, the study of markers of stem cell (OCT4, SOX2) in colon cancer and the relationship between the expression of these genes and their clinical outcomes have particular importance. Patients with tumor recurrence after the chemotherapy for CRC show a higher level of expression of these genes (SOX2, OCT4) rather than those who do not have. The increased levels of these genes are clearly associated with poor prognosis. Gene expression of OCT4 and SOX2 can predict recurrence of the tumor and improve the prognosis of patients with rectal cancer treated with preoperative chemoradiotherapy. These genes together could be associated with recurrence and metastasis after chemotherapy.[8]

RT-PCR analysis of cells engaged in colon cancer showed gene expression of OCT4 and SOX2 and also IHC analysis confirmed the nuclear and cytoplasmic expression of OCT4. The role of OCT4 and SOX2 in colon cancer has been confirmed and suggested the level of gene expression could be defined 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.[9]

Moreover, SOX2 is involved in metastases of lymph nodes. Also, these findings show the importance of markers associated with stemness in detecting the high risk colon cancer for distant metastases.[10]

Gene expression of OCT4 and SOX2 as self-renewal genes in colorectal adenocarcinoma HT-29 and $CaCO_2$ as cancer cells is confirmed that it helps to confirm the cancer stem cell theory. However, further studies are needed to find the cause of the existence of such localization.[11]

Given that, it is clinically proven that colon polyps can be converted to adenocarcinoma but in the case of pathogenesis of adenocarcinoma, a variety of content has been mentioned. But one of the causes of cancer could be related to the gene expression of stemness in cells, and studies about the role of SOX2 and OCT4 gene on polyps has been rare so far.

Therefore, this study, in the first stage, determine the relationship between these genes and cancer and in the second stage responds to this question of whether the emergence of dysplasia in colorectal polyps compared with healthy tissue, is related to these genes or not; In other words, whether the neoplastic and stepped progress of tumor from healthy to dysplasia and from dysplasia to cancer which is provable by morphological method, is compliant with to the level of gene expression of these genes in terms of quality and quantity or not?

MATERIALS AND METHODS

This is a cross-sectional study. In order to prepare the colon tissue sample, 20 patients with symptoms such as bleeding or severe anemia and the result of colonoscopy and biopsy reported as adenocarcinoma undergoing colectomy and had simultaneous polyps and cancer in their macroscopic examination in the laboratory have been participated. It should be noted that where microscopic polyps had dysplasia, they were included in the study. No limitation has been considered with regard to the stage of the disease. For each patient, a sample of healthy tissue, dysplastic polyp tissue (tubular, villous, or tubulovillous), and colon adenocarcinoma were provided as microscopic sections and staining on each tissue was performed through IHC method by markers OCT4 (mouse monoclonal anti-OCT4, Diagnostic BioSystem, Clone ≠ NRG1.1) and SOX2 (mouse monoclonal anti-SOX2, Diagnostic BioSystem, Clone ≠ NRG5.6) as follows. First, the slides were put in tumor at 74°C for 50 min to embed paraffin, and then they were put in two containers with xylenol (5 min), absolute alcohol (5 min), and 96% alcohol (2 min), respectively. For antigen retrieval, the slides were put on citrate buffer with pH = 6 in boiling water bath for 1 h. Then, the slides were put in phosphate buffer and then in 3% hydrogen peroxide for 10 min and were re-washed with phosphate buffered saline (PBS). Primary antibodies were poured on slides for 60 min, and the slides were placed in PBS and then put in EnVision Dual Link System Peroxidase for 1 h. The slides were washed again in phosphate buffer and put in 3, 3'-diaminobenzidine slides for 3–5 min (corrosion) and then, washed with distilled water. Finally, the slides were put

into hematoxylin and dewatering process (taking on alcohol) was performed. By putting a cover slip and pasting slides by special IHC, staining was performed [Figures 1 and 2]. The percentage of stained cells with nuclear pattern (with ranking of Grade 0: Negative, Grade: 1–25%, Grade II: 26–50%, Grade III: 51–75%, and Grade IV: 76–100%) in each healthy sample, polyp, adenocarcinoma per high power field (averagely) and the intensity of their staining (with ranking of 0: Negative, 1: Weakly positive, 2: Mod (inter) positive, 3: Strongly positive) were recorded. (Note: In each cycle of doing IHC, positive control [seminoma for OCT4 and squamous cell carcinoma larynx for SOX2] and for both marker and the negative control, all conditions were kept the same, except that the primary antibody was omitted). The collected data were interred into SPSS version 18.0, (SPSS Inc., Chicago, IL, USA) software and for analysis, Chi-square test, independent *t*-test, and linear regression test were run and the level of significance was considered as <0.05.

RESULTS

The study sample consisted of 20 patients including 11 men (55%) and 9 women (45%) with a mean age of 55.6 ± 9.88 among which 3 of them (15%) were from Afghanistan. Pathology results showed that tubular polyp was the most common types of polyps (60%) and the most common site of polyps was sigmoid with 8 cases (40%) and the maximum size of 1.83 ± 0.81 mm and the number was at least 1 and a maximum of 4. On the other hand, the cancer location in 9 patients (45%) had the highest prevalence in sigmoid with the cancer stages of I to IV. Metastatic lymph nodes were eventually positive in 11 cases (55%) [Table 1].

In order to detect the relation between these genes and development of colon cancer, SOX2 and OCT4 gene expression study in development of dysplasia in the colon polyps and cancer showed that in SOX2 marker, the percentage of stained cells and color ability of normal tissue to dysplasia and from dysplasia to carcinoma increased significantly so that the degree of expression and staining intensity had the lowest and the highest levels in normal and cancer tissues respectively ($P < 0.05$). But OCT4 marker had no expression in the three studied tissue samples and therefore it can say the diagnostic power of the gene is not enough to identify cancer. So in general, the SOX2 gene expression power is more than OCT4 that this difference was significant only in normal tissue ($P = 0.035$) [Table 2].

Also, expressed by markers SOX2 in comparison with the intensity of staining in tissue showed that in normal tissue from 4 cases have been 3 cases of intensity 1 and 1 cases of intensity 2. Against in polyp tissue, frequency in intensity 2 has 3. And eventually cancer tissue from 6 expressions observed in 5 cases were with the highest intensity (intensity 3), in fact, the progress of the disease, the staining intensity of marker been effective ($P < 0.05$) [Table 3].

On the other hand, according to the performed regression analysis, no factor played an effective role in the expression of SOX2 in normal tissue; but polyp tissue the polyp type had a significant effect on the percentage of stained cells and in the cancer tissue, the role of race and lymph node with the impact factor of 2.589 and 0.318 were effective in the gene expression so that the percentage of stained cells and the intensity of staining in an Afghanistan patients and someone who had lymph node were significantly stronger

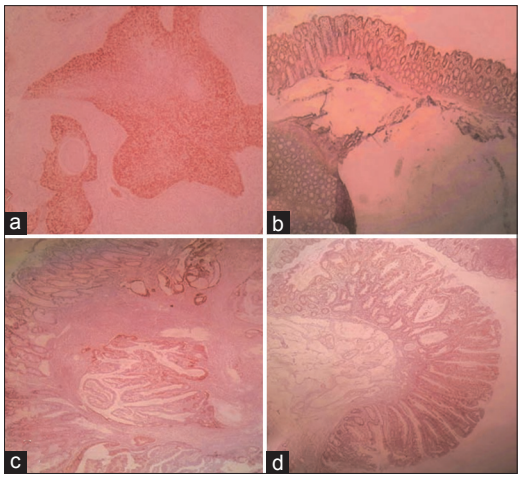

Figure 1: Immunohistochemical staining of SOX2; (a) Positive control sox2 squamous cell carcinoma, (b) Sox2 immunohistochemistry in normal, (c) Sox2 immunohistochemistry in adenocarcinoma of colon, (d) Sox2 immunohistochemistry in villus polyp

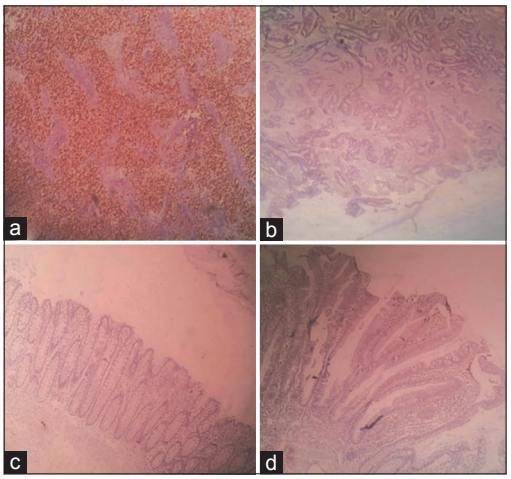

Figure 2: Immunohistochemical staining of OCT4; (a) Positive control of OCT4 (seminoma), (b) negative immunohistochemistry staining for oct4 in adenocarcinoma of colon, (c) OCT4 immunohistochemistry staining in normal epithelium of colon, (d) OCT4 immunohistochemistry staining in villus polyp

Table 1: Demographic and pathological characteristics patient

Variable	*n* (%)
Number of patients	20
Age (mean±SD)	55.6±9.88
Sex	
Male	11 (55)
Female	9 (45)
Type of polyp	
Tubular	12 (60)
Villous	5 (25)
Tubulovillous	3 (15)
Maximum size of polyp (mm)	1.83±0.81
Polyp site	
Descending colon	3 (15)
Sigmoid	8 (40)
Rectum	4 (20)
Increasing colon	3 (15)
Transverse	2 (10)
Stage of cancer	
I	1 (5)
II_A	5 (25)
II_B	3 (15)
III_A	4 (20)
III_B	2 (10)
III_C	3 (15)
IV	2 (10)
Race	
Iranian	17 (85)
Afghan	3 (15)
Number of polyps	
1	10 (50)
2	4 (20)
3	3 (15)
4	2 (10)
Cancer site	
Descending colon	2 (10)
Sigmoid	9 (45)
Rectum	4 (20)
Increasing colon	3 (15)
Transverse	2 (10)
Metastatic lymph node	
–	9 (45)
+	11 (55)

SD: Standard deviation

Table 2: Frequency distribution of the percentage and intensity of stained cells in Sox2 and Oct4 markers

Variables	Normal tissue (%)	Polyp tissue (%)	Cancer tissue (%)	*P**
Grade of Sox2				
0	16 (80)	16 (80)	14 (70)	0.040
I	4 (20)	1 (5)	0 (0)	
II	0 (0)	3 (15)	2 (10)	
III	0 (0)	0 (0)	3 (15)	
IV	0 (0)	0 (0)	1 (5)	
Intensity of Sox2				
0	16 (80)	16 (80)	14 (70)	0.018
1	3 (15)	1 (5)	1 (5)	
2	1 (5)	3 (15)	0 (0)	
3	0 (0)	0 (0)	5 (25)	
Grade of Oct4				
0	20 (100)	20 (100)	20 (100)	-
I	0 (0)	0 (0)	0 (0)	
II	0 (0)	0 (0)	0 (0)	
III	0 (0)	0 (0)	0 (0)	
IV	0 (0)	0 (0)	0 (0)	
Intensity of Oct4				
0	20 (100)	20 (100)	20 (100)	-
1	0 (0)	0 (0)	0 (0)	
2	0 (0)	0 (0)	0 (0)	
3	0 (0)	0 (0)	0 (0)	

*Level of significance of comparison among three tissue types with the percentage and intensity of cells stained at each marker

than that in Iranian patient and without lymph node ($P < 0.05$); also, as it can be seen in Figure 2, the rate of expression in villous polyps with two cases was more which is statistically was significant. On the other hand all observed expression in third stages of C and A was cancer; however, these factors and others had statistically no significant role in the expression and incidence of gene [Table 4 and Figure 3].

DISCUSSION

CRC is one of the fastest worldwide growing and the most common causes of death cancers in which the life expectancy is slightly reduce, especially in young adults,[12] and has two major subtypes including high-under graded B-catenin cells caused by lesion in the Wnt/adenomatous polyposis coli/B-catenin pathway and microsatellite instability cells, leading to disrupt of from stem cells to normal colorectal epithelial cells transformation.[13] Both of these pathways are in association with pluripotency transcription factors such as Sox2, and Oct4. Furthermore, CRC stem cells have several markers such as CD133 and CD44, which are very important in order to distinct from other normal cell phenotypes.[14] CD133 expresses co-related with an expression of Sox2 and Oct4.[12] CD44 also promotes pluripotency by co-operating with Sox2 and Oct4.[15]

In the present study, we investigated about the potential tumor markers in the prognosis of CRC. We couldn't find any significant association between the expression of Oct4 and kinds of tissues (normal, polyp, and cancer) for both intensity and number of stained tissue cells. In the normal adult cells, Oct4 is not expressing normally,[16] but with not presence of expression in polyp or tumor tissues, we think that due to the limitation of our study population, we couldn't

find any expression of Oct4 protein. In the previous study, it has been found that inhibition of Oct4 in colon cancer cells was beneficial for preventing them from metastasis.[17]

Table 3: Comparison the frequency distribution of percentage of stained cells and the intensity of staining in the three tissues studied

Tissue	Grade	Intensity (%)				*P*
		0	1	2	3	
Normal	I	-	3 (75)	1 (25)	-	0.046
Polyp	I	-	1 (100)	-	-	
	II	-	-	3 (100)	-	
Cancer	II	-	1 (50)	-	1 (50)	
	III	-	-	-	3 (100)	
	IV	-	-	-	1 (100)	

Table 4: Regression analysis of the role of factors affecting the percentage of cells stained in markers Sox2

Factors	Grade Sox2 in normal tissue		Grade Sox2 in polyp tissue		Grade Sox2 in cancer tissue	
	β	*P*	β	*P*	β	*P*
Age	0.021	0.169	−0.014	0.325	0.007	0.688
Sex	0.156	0.488	0.197	0.371	0.073	0.789
Race	−0.014	0.979	−0.106	0.838	2.859	0.001*
Size of polyp	-	-	−0.011	0.943	-	-
Type of polyp	-	-	0.541	0.040*	-	-
Count of polyp	-	-	−0.008	0.933	-	-
Location polyp	-	-	−0.024	0.930	-	-
Stage of cancer	-	-	-	-	0.001	0.636
Location cancer	-	-	-	-	−0.063	0.859
Lymphnode	-	-	-	-	0.318	0.038*

*: Effective Factors in the significance level of less than 0.05

Moreover, Oct4 could regulate epithelial-mesenchymal transition, and lack of its expression might inhibit the epithelial to mesenchymal transition pathway, which consequently impedes from migration and invasion of CRC cells.[17] As the result, we presumed that our patients were not in metastasis phase, but our result indicated that more than 50% of our population had metastatic lymph node. It might be due to the lack of other markers' assessment such as EpCAM, because of its interaction with β-catenin in c-myc transcription,[18] or prevent p53 activity[19] and Lgr5 (since promoting Wnt signaling pathway to transcription of β-catenin),[20] which leads to undistinguishing reason for not having expression of Oct4; therefore, we could not find the alternating tumor initiation pathways to express the persuasive explanation. Also, according to the previous investigations, Oct4 is good marker for prognosis of CRC,[21] and expression of Oct4 informed the adverse prognosis of CRC patients,[14] but we could not find Oct4 expression in different patients' tissues and would not discuss it as well.

Despite not having Oct4 expression in our population, our assessment in accordance with previous study[22] revealed that the percentage of cells expressing Sox2 from normal tissues of patients to cancer tissues increased (P = 0.040). Meanwhile, the intensity of IHC stain from normal to cancer tissues significantly increased (P = 0.018). Sox2 is contributing to the cancer progress through its functional control of the pluripotent state and cell self-renewal.[23,24] Besides, expression of Sox2 is contributing to keep stemness features of tumor-initiated cells, which has a pivotal role, in

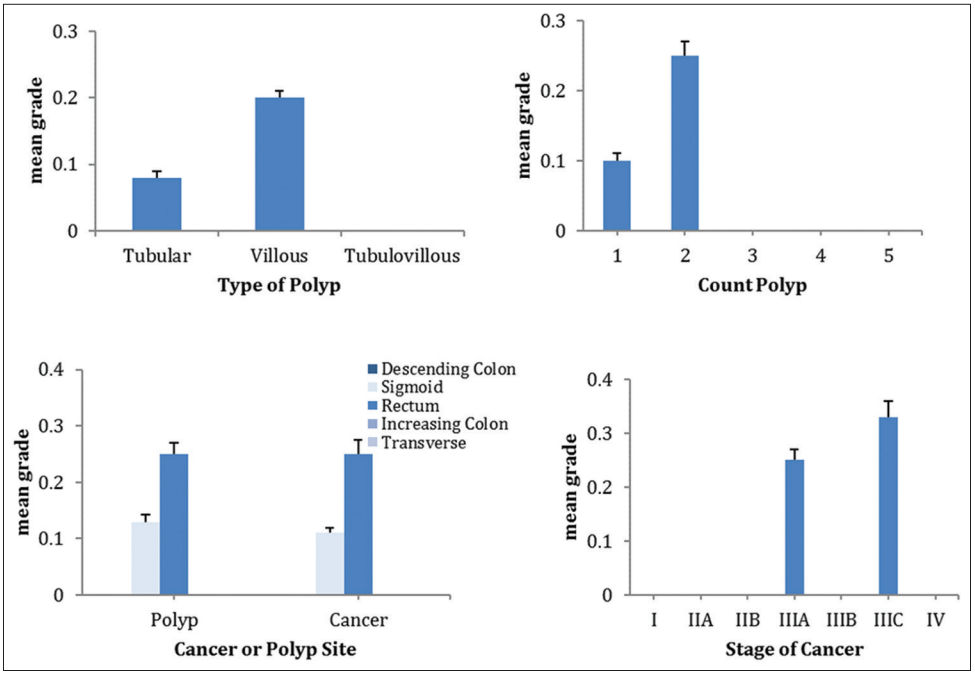

Figure 3: The comparative investigation of the mean expression of gene in terms of type, number, polyp site, and cancer stage

tumorigenic properties.[25,26] In addition, evaluating of Sox2 expression in both nuclear and cytoplasm of CRC cells have demonstrated the enhanced level of protein, of which enhances the ability to colony formation, tumor development, and migration features.[27]

Another point we have found was that expression of Sox2 increased from normal to cancer tissue ($P = 0.046$). Other studies have shown that expression of Sox2 protein was dramatically increased in cancer cells compared to normal tissues,[27] and it was occurred because of inducing cancer stemness in CRC cells,[28] and controlling many receptor mediating signaling pathways participates in CRC progress such as epidermal growth factor receptor, which is one of the most important therapeutic targets of CRCs.[29,30]

Interestingly, Grade of Sox2 expression was been in association with the type of polyp. One the other word, grade of Sox2 expression in villous polyp was higher than that of in Tubular polyp. It has been found that villous polyp due to the vast surface area and its expansion into the lumen has the highest potential for being malignant.[31] Furthermore, according to our study, the race had a significant correlation with Sox2 expression ($P < 0.001$). Also, in accordance with the previous investigation,[32] the expression of Sox2 in the patients with metastatic lymph node was significantly higher than those who not had any infected lymph node. Another study showed that patients with tumors with higher Sox2 staining by IHC developed more often metastases to lymph nodes and liver.[33]

CONCLUSION

This study demonstrated the noticeable insight for the current study. We could not find any association between Oct4 and CRC patients but as it illustrated, there was a significant correlation between Sox2 expression and CRC patients in many aspects such as race, type of polyp, presence of lymph node, grade and intensity of Sox2 in different types of patients' tissues. Regarding our findings, the expression of Sox2 would be a liable marker for evaluating of cancer progression and could be a treatment target of CRC cells.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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