Immunohistochemical and Tissue Array Study for Comparison of the Expression of Tumor Suppressor Genes and with Intercellular Adhesive Molecules in Colorectal Adenocarcinoma and Nontumoral Colon

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Introduction: Colorectal Carcinoma is a main health problem in many countries and the third common cancer in Iran. This malignancy at present is the most curable carcinoma of gastrointestinal tract. Variation in the expression of the proteins produced by P_{53}, P_{21}, P_{16}, E-cadherin, and β-catenin genes have been noted in this malignancy and may be important in the prognosis and therapeutic response rate. The aim of this study was to compare the frequency and pattern of expression of these proteins in tumoral and nontumoral colonic mucosa. The correlation with prognostic factors including tumor stage, grade, and vascular and perineural invasion was also determined.

Material and Methods: The paraffin blocks from tumoral and nontumoral parts of the colon obtained from 58 patients with colorectal adenocarcinoma were studied along with 50 colectomic cases in individuals without malignancy. Cylindrical tissue fragments were obtained from appropriate parts of donor blocks by using a 2.5 mm punch biopsy instrument. Each 30 samples were manually arrayed in one tissue array block. Expression of above genes was investigated after sectioning the blocks and immunohistochemical staining of slides.

Results: The expression of P_{53} in tumor cells was significantly more common than in colonic nontumor cells and colon of individuals without tumor ($p<0.001$); expression of this protein in tumoral tissues was directly related to vascular invasion ($p=0.017$). The expression frequency of P_{21} and P_{16} in tumor cells was less than nontumoral tissues of patients with cancer and patients without cancer ($p<0.001$). These two gene products showed no correlation with prognostic factors. The expression frequency of membranous E-cadherin and β-catenin in tumor cells was not different from controls, while the membranous expression of E-cadherin was inversely related to cell differentiation ($p=0.023$) and vascular invasion ($p=0.025$). In addition, the membranous expression of β-catenin was inversely related to vascular invasion ($p=0.049$). Cytoplasmic and nuclear expression of β-catenin in tumor cells were significantly higher than their expression in the controls ($p<0.001$). Cytoplasmic expression of this marker was inversely related to disease stage ($p=0.013$), while its nuclear expression was inversely related to cell differentiation ($p=0.012$).

Conclusion: According to our data, it seems that we are able to predict aggressive capacity of the colorectal tumor by determining the frequency and pattern of expression of P_{53}, E-cadherin and β-catenin proteins. These studies can be done simply on formalin-fixed small biopsy samples before surgery to provide valuable information for surgeons, gastroenterologists, and oncologists to choose the best therapeutic approach and predict the therapeutic response. Manual tissue array method is believed to be an economical technique for similar research projects.

Keywords: Colorectal adenocarcinoma, Immunohistochemistry, Tissue array, P_{53}, P_{16}, P_{21}, E-cadherin, beta catenin

Introduction

Colorectal carcinoma is a malignant epithelial tumor of large intestine with glandular growth pattern (1). In USA, colorectal carcinoma is the forth common malignancy after breast, prostate, and lung cancer, and also the most common and curable malignancy of gastrointestinal tract (2). It is the third common cancer in Iran (3). Adenocarcinomas constitute about 98% of all large bowel malignancies (4, 5). The peak of incidence is between 60-79 (mean 62) years old (1, 5, 6). Genetic, environmental, and dietary factors and pelvic irradiation have all been implicated in the appearance of this carcinoma (1). Most cases occur sporadically (5). Molecular carcinogenesis in colorectal adenocarcinoma includes two separate pathways: APC/ beta-catenin pathway (containing mutation of APC tumor suppressor gene, mutation of beta-catenin, k-ras, P_{53}, and SMAD4 and telomerase activity) and microsatellite instability pathway or genetic defect in DNA mismatch repair genes. Mutation in E-cadherin, beta-catenin and Von-Hippel-Lindau genes are known findings (1, 3). Wild type P_{53} has several roles in cell cycle; it causes cell cycle arrest at the end of G_1 phase via induction of P_{21} waf_1/ Cip_1, induces transcription of GADD45 involved in DNA repair, and finally induces apoptosis via BAX gene (a Bcl_2 inhibitor) (1, 7). Wild P_{53} is not detectable by IHC (because of short half life of about 20 min). Mutant P_{53} has lost its ability to suppress cell proliferation, has long half life (6h), and is detectable by IHC in nucleus (1). Tumor cells containing mutant P_{53} are resistant to apoptosis induced by radiotherapy and chemotherapy (1). P_{21} waf_1/ Cip_1 is a tumor suppressor gene that its synthesis is induced by P_{53} in response to DNA damage, inhibits cyclin D-CDK_4 complex, and causes cell cycle arrest. It also causes cell cycle arrest in G_2 phase independent to P_{53} (1, 7). Wild P_{21} is detectable by IHC in nucleus (1, 7). P_{16} INK 4a is a tumor suppressor gene that inhibits cyclin D-CDK 4 complex and causes cycle arrest (1, 7). Wild P_{16} is detectable by IHC in nucleus (1). E-cadherin is a transmembrane glycoprotein that causes intercellular adhesion and is detectable by IHC ic cell membrane (1, 7). Beta–catenin which is normally located at cell membrane can activate growth stimulators (C-myc & cyclin D) by transferring them into cytoplasm and then to nucleus (1, 7).

Tissue array is an array of ten to hundreds of thin cylindrical tissue fragments of various origins in one paraffin block that enables simultaneous analysis of various tissues and controls in the same conditions and saves the time, labor, and costs (8-14).

The aim of this study was to compare the frequency and pattern of expression of these proteins in tumoral and nontumoral colonic mucosa of patients with and without this malignancy. The correlation with prognostic factors including stage, grade, vascular, and perineural invasion was also determined.

Material and Methods

This research was performed in Dr. Shariati Hospital between 1380-3. Cases were selected from paraffin embedded blocks of tumoral colons of patients with a history of colectomy due to colorectal adenocarcinoma (State 1 or S1). Two different control groups were selected: control 1 (State 2 or S2) including paraffin-embedded blocks of nontumoral colon the patients whose tumors had been selected for S1, and control 2 (State 3 or S3) paraffin-embedded blocks of colectomized patients not affected by adenoma, adenocarcinoma, and IBD (eg. trauma, ischemia, and etc.). Cases with extensive areas of necrosis or hemorrhage were excluded from research (15, 16, 17). The pathology reports were reviewed to find cases and controls. The slides were selected and reviewed. The corresponding paraffin blocks were selected and the areas of interest were marked.

The areas of interest were removed from paraffin blocks by using a disposable 2.5 mm biopsy punch. Selected samples were kept in separate labeled containers. The tissue array moulds, consisting of two pieces of L-shaped metal, were inserted in a microbiology plate to inhibit the paraffin leakage.

The combination of mould and plate were filled with warm liquid paraffin and kept warm during arraying of samples. Thirty core samples including 27 cases and controls and 3 positive controls of IHC markers were arrayed in each block (Figure 1).

The prepared blocks were sliced and the slides were stained by H & E method, and if optimal, by IHC method (Figure 2).

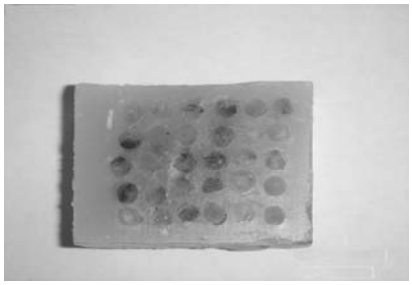

Fig 1

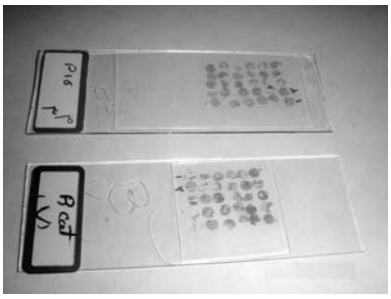

Fig 2

Presumptive positive IHC controls were selected based on previous studies (15-32) and IHC marker catalogues. Thirty cylindrical pieces of suspected tissues were arrayed in one tissue array block that was sliced and stained by IHC method for five markers.

Positively stained sampled were chosen, including uterine cervical squamous cell carcinoma (SCC) for P_{21}, breast intraductal carcinoma (IDC) for P_{53} and P_{16}, and normal gastric epithelium or parathyroid adenoma for E-cadherin and beta-catenin.

The IHC results were classified according to staining severity as 0 = negative, 1 = 1+, 2 = 2+, and 3 = 3+, and based on staining extension as 0 = less than 5%, 1 = 5-25%, 2 = 26-50%, 3 = 51-75%, and 4 = 76-100% of cells. Final score was equal to severity×extension, as follows: 0-4= negative or markedly decreased and 5-12 = positive expression. The results were statistically analyzed by SPSS software and examined by Fisher's exact test or Pearson chi–Square test where needed.

Results

Fifty eight blocks were found for S1, 58 for S2, and 50 for S3.

Sex distribution in cases and S2 controls was 51.7% male and 48.3% female, while it was 70% male and 30% female in S3 controls. The mean age of cases and S2 controls was 49.55±13.71 (20-75) years, while it was 55.66±17.34 (6-85) years in S3 controls. Tumor type in subjects was as follows: NOS 81.9%, mucinous 8.6%, signet ring 3.4%. Location of tumors has been shown in Table 1.

Table 1: Location of tumor.

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	1.00	14	24.1	24.1	24.1
	2.00	6	10.3	10.3	34.5
	4.00	2	3.4	3.4	37.9
	5.00	14	24.1	24.1	62.1
	6.00	9	15.5	15.5	77.6
	7.00	10	17.2	17.2	94.8
	8.00	1	1.7	1.7	96.6
	9.00	2	3.4	3.4	100.0
	Total	58	100.0	100.0	

a STATE = 1.00

1. Cecum, 2. Ascending colon, 3. Transverse colon, 4. Descending colon, 5. Sigmoid colon, 6. Rectosigmoid colon, 7. Rectum, 8. Anal canal, 9. Not specified

Table 2: Results of comparison between gene expression in various states.

Marker	Difference in state 1 with State 2		Difference in state 1 with State 3		Difference in state 2 with State 3	
P_{53}	YES $P_{value<0.001}$	Positivity in $S_1>S_2$	YES $P_{value<0.001}$	Positivity In $S_1>S_3$	No	Negativity in $S_2=S_3$= 100 %
P_{21}	YES $P_{value<0.001}$	Negativity in $S_1>S_2$	YES $P_{value<0.001}$	Negativity in $S_1>S_3$	No	
P_{16}	YES $P_{value<0.001}$	Negativity in $S_1>S_2$	YES $P_{value<0.001}$	Negativity in $S_1>S_3$	No	
E-cadherin	No		No		No	
β-catenin M	No		No		No	Positivity in $S_2=S_3$= 100 %
β-catenin C	YES $P_{value<0.001}$	Positivity in $S_1>S_2$	YES $P_{value<0.001}$	Positivity in $S_1>S_3$	No	
β-catenin N	YES $P_{value<0.001}$	Positivity in $S_1>S_2$	YES $P_{value<0.001}$	Positivity in $S_1>S_3$	No	

Table 3: Summary of gene expression in correlation with histologic prognostic factors

IHC Marker	Stage Fisher's exact test P_{value}	Grade Pearson chi square P_{value}	Vascular Invasion Fisher's exact test P_{value}	Perineural Invasion Fisher's exact test P_{value}	Tumor Type Pearson chi square P_{value}
P53	1	0.392	0.017	1	0.506
P21	0.783	0.255	0.124	0.700	0.954
P16	0.729	0.220	0.710	1	0.374
E-cadherin	1	0.025	0.023	0.418	0.106
β–catenin M	0.130	0.842	0.049	1	0.044
β -catenin C	0.013	0.038	0.553	1	0.205
β -catenin N	0.284	0.012	0.767	1	0.416

β-catenin M=Membranous
β-catenin C=Cytoplasmic
β-catenin N=Nuclear

Summary of results of gene expression in various states has been shown in Table 2. Correlation between gene expression and prognostic factors is summarized in Table 3.

Discussion

The P_{53} expression in tumor cells was significantly more common than non-tumoral colonic cells of patients with and without colorectal adenocarcinoma (p<0.001). The expression of P_{21} & P_{16} in tumor cells was significantly less common than non-tumoral colonic cells of patients with and without colorectal adenocarcinoma (p<0.001). The frequency of membranous expression of E–cadherin and beta–catenin were not significantly different in three states. Cytoplasmic and nuclear expression of beta–catenin in tumor cells were significantly more common than non–tumoral colon of subjects with and without colorectal adenocarcinoma (p<0.001). The expression frequency of none of the proteins in non-tumoral colon of patients with colorectal adenocarcinoma was significantly different from unaffected individuals. This finding may be important in distinguishing tumoral from non-tumoral border in tumor resection. The P_{53} expression in colorectal adenocarcinoma cells was directly related to vascular invasion (p=0.017).

The frequencies of P_{21} & P_{16} expression in tumor cells were not related to prognostic factors. The frequency of membranous expression of E–cadherin was inversely related to cell differentiation (p=0.023) and vascular invasion (p=0.025). Frequencies of Cytoplasmic and nuclear expression of beta-catenin was inversely related to vascular invasion (p=0.049). The frequency of cytoplasmic expression of beta-catenin was inversely related to disease stage (p=0.013).

The frequency of nuclear expression of beta-catenin was inversely related to cell differentiation (p=0.012).

Overall, overexpression of P_{53}, underexpression of P_{21} & P_{16}, and shift of beta–catenin expression from cell membrane to cytoplasm and nucleus in tumor cells were seen more than those in non-tumor colonic cell of patients with and without adenocarcinoma. Expression of membranous E–cadherin and nuclear beta-catenin were directly related to cell differentiation. Vascular invasion was directly associated with P_{53} expression and inversely related to membranous expression of E–cadherin and beta–catenin. Perineural invasion was related to none of above markers.

In study of Valassiadou KE *et al*. in 1997 (20) and Goussia AC *et al.* in 2000 (21), P_{53} overexpression was inversely related to probability of lymphatic invasion and aggressive potential, as opposed to our study. According to Hirvikoski P *et al.* in 1999 (22) and Acolin A *et al.* in 2005 (23), mutation or overexpression of P_{53} was not correlated to prognosis. Diez M *et al.* in 2005 (24) showed that P_{53} was the only predictive of high recurrence risk in a subgroup of patients with stage 3 tumors. Mitomi M *et al.* in 2005 (25) showed that down-regulation of P_{21} was associated with lymph node and/or liver metastasis. In a study performed by Yasui W *et al*. in 1997 (26), P_{21} expression was inversely

associated with invasion to deeper than muscularis propria and tumor progression. Tada T *et al*. in 2003 (27) showed that colorectal cancers with reduced P_{16} expression had more aggressive potential of lymphatic infiltration and tumor progression.
In our study, reduced expression of P_{21} & P_{16} was seen in tumoral cells compared to non-tumor cells, while it was not correlated to prognostic factors.
In studies performed by Herter P *et al.* in 1999 (28), Utsunomiya T *et al.* in 2001 (29), and Delektorskaya W *et al.* in 2005 (30), cytoplasmic and nuclear translocation of beta-catenin expression from cell membrane was seen in tumor cells, which was associated with aggressive potential in agreement with our study. In studies performed by Sloncova E *et al*. in 2001 (31) and Jesus EC *et al*. in 2005 (32), expression of E–cadherin was not related to prognostic factors, as opposed to our study which showed inverse relationship between frequency of membranous expression of E-cadherin, and cell differentiation (p=0.023) and vascular invasion (p=0.025).
Colorectal adenocarcinoma is the most common and most curable malignancy of the gastrointestinal tract. Its pathogenesis is influenced by environmental and genetic factors. Genetic factors include proteins that regulate cell replication cycle including P_{53}, P_{21}, and P_{16} tumor suppressor genes, an adhesive molecule called E-cadherin, and beta-catenin which regulates cell cycle and mediates beta–catenin action. Expression of these proteins has been studied in multiple separate studies, which have shown correlation with prognosis and responsiveness to the therapeutic methods in patients with colorectal adenocarcinoma.
In this study, we tried to solve the technical problems of tissue array and immunohistochemical staining methods to compare the expression of described proteins in tumor cells of colorectal adenocarcinoma with colonic non-tumor cells of patients with and without adenocarcinoma.
Tissue array method saves morphologic characteristics of tissue samples and saves in time, labor, and cost.
In this study, increased expression of P_{53}, decreased expression of P_{21} and P_{16}, and shift of beta-catenin expression from cell membrane to cytoplasm and nucleus were seen in colorectal adenocarcinoma cells (but not in adjacent colonic cells) as compared to the colon of unaffected people. Cytoplasmic expression of beta-catenin was directly associated with disease stage. In addition, membranous expression of E-cadherin inversely related to tumor grade, while nuclear expression of beta–catenin was directly related to it. Vascular invasion was accompanied by increased expression of P_{53}, and decreased membranous expression of E-cadherin and beta-catenin. Perineural invasion was not associated with any change in expression of above proteins.

Conclusion

The aggressive capacity of colorectal adenocarcinoma can be predicted by determining the frequency and pattern of expression of P_{53}, P_{21}, P_{16}, E-cadherin, and beta-catenin proteins. These studies can be done simply on formalin–fixed small biopsy samples, probably providing reliable information for surgeons, gastroenterologists, and oncologists to select the best therapeutic approach and predict therapeutic response rate. Expression of these markers may be helpful in marking of tumoral and non-tumoral areas of tumoral colons. The tissue array method enables multiple cores to be analyzed simultaneously and in the same conditions and so significantly saving the time, labor, and costs.
The manual method of tissue array is an economical method compared to automated tissue array and can be used as a technique for future diagnostic applications and researches.

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