

Does glucagon like peptide-2 receptor expression have any effect on the development of human colorectal cancer?

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Background/aims: Glucagon like peptide-2 may play an important role in human colon cancer and polyp development because of its proliferative and antiapoptotic effects especially in colon. In this study, we investigated the role of human glucagon like peptide and it's receptor in development of human colorectal carcinogenesis **Material and Methods:** The study includes 30 patients in colon cancer group and 20 patients in colonic polyp group who have been diagnosed by endoscopic and pathologic examination in Dokuz Eylül University, Department of Gastroenterology within 2 year-period. For comparison biopsies were taken from normal appearing colonic mucosa of the same patient. The cancer, polyp and normal colon mucosa samples were stained with glucagon like peptide receptor antibody by immunohistochemical method. **Results:** Glucagon like peptide - 2 receptor positivity of colon cancer patients was 20 % (6/30) in focal cytoplasmic coloration while it was 0 % in colonic adenomas and 100 % in enteroendocrine cells of normal colonic mucosa. Statistically significant differences were found by the comparison of colonic polyp and normal colonic tissue ($p=0.000$), colonic cancer and normal colonic tissue ($p=0.000$) and colonic polyp and cancer tissues ($p=0.023$). **Conclusion:** Glucagon like peptide-2 receptor expression in colonic adenomas was not detected in human in contrary to the study on mice. Our study suggested that Glucagon like peptide-2 receptor expression is not a factor in adenoma-cancer pathogenesis. More studies are needed on this subject with more facts and different methods.

Key words: Glucagon like peptide-2, colon polyp, colorectal cancer

Kolon kanseri ve polip dokusunda glukagon like peptid-2 Reseptör

Amaç: İnsanlarda kolon kanseri ve bunun öncüsü kolon poliplerinin oluşumu ile glukagon like peptid-2 (GLP-2) ve glukagon like peptid-2 reseptör arasındaki ilişkiyi göstererek kolon polip ve kanseri gelişim patogenezine katkıda bulunmaktadır. **Yöntem:** Alt gastrointestinal sistem endoskopisi esnasında kolon polibi saptanıp biyopsi alınan ve yine endoskopik işlemde kolon kanseri ile uyumlu bulguları saptanıp biyopsi alınan hastalardan, patolojik tanıları kolon polibi ve kolon kanseri olan, kolon polibi grubuna 20 hasta (12 E, 8 K, ortalama yaş: 64 ± 9.4); kolon kanseri grubuna 30 hasta (17 E, 13 K, ortalama yaş: 62 ± 10.7) alınmıştır. Aynı hastalardan, çalışmanın karşılaştırma verilerini oluşturabilmek için normal kolon mukoza dokularından da biyopsi alınmıştır. Poli-L-lizininli lamlara hazırlanan kesitler glukagon like peptid-2 reseptör antikor (1:100-1:200, 1 mg/ml) immunhistokimyasal boyasıyla boyanmıştır. **Bulgular:** Glukagon like peptid-2 reseptör pozitifliği; kanserli hastalarda %20 (6/30) oranında fokal sitoplazmik boyanma şeklinde, adenomlarda %0, normal mukozadaki enteroendokrin hücrelerde %100 bulunmuştur. Polip ile normal ($p=0.000$) ve kanser ile normal doku ($p=0.000$) karşılaştırıldığında istatistiksel anlamlı fark saptanmıştır. Polip ile kanser dokusu karşılaştırıldığında da istatistiksel anlamlı fark saptanmıştır ($p=0.023$). **Sonuç:** Sonuç olarak; farelerde yapılan çalışmanın aksine insanda kolon poliplerinde glukagon like peptid-2 reseptör ekspresyonu gösterilmemiştir. Bu; kolon adenom-kanser patogenezinde glukagon like peptid-2 reseptör ekspresyonunun etkin olmadığını düşündürmektedir. Ancak ileri evre kolon kanseri ve olasılıkla metastaz patogenezinde, glukagon like peptid-2 reseptör ekspresyonunun etkisi olabilir. Bu konuda daha fazla olgu sayısı ve farklı metodlarla yapılacak yeni çalışmalara ihtiyaç vardır.

Anahtar kelimeler: Glukagon like peptid-2, kolon adenom, kolon kanseri

INTRODUCTION

Colorectal cancer (CRC) is an important problem with varying frequency in different societies of the world. The prevalence of CRC ranks first among all malignancies. In the United States of America (USA), where the statistical data are reliable, CRCs rank third (in both sexes) after prostate and lung cancer in men and after breast and lung cancer in women (1). In cancer-related mortality, CRC is ranked second after lung cancer (1).

The majority of CRCs are sporadic. However, genetic and environmental factors are contributory, and cell oncogenes, growth factors and receptors are also cited as playing a role in the development and growth of CRCs (2). Most of the adenoma structures of the large bowel extend to the lumen, then called polyp, and there are strong data showing that carcinomas generally arise from adenomatous polyps.

Glucagon-like peptide (GLP)-2 is a 33 amino acid peptide hormone released from intestinal endocrine cells following nutrient ingestion. GLP-2 is mainly produced and released from enteroendocrine L cells, which are localized in the small and large bowel (3). Its intestinotrophic effect in epithelial mucosa can be explained via increasing crypt cell proliferation and inhibition of apoptosis.

The proliferative effect of GLP-2 was shown in mice, rats, pigs, and humans by giving exogenous GLP-2 (4). Similar studies on animals show that GLP-2 accelerates the mucosal healing in chemotherapy-induced enteritis and indomethacin-induced colitis models (5). Increased GLP-2 levels and decreased DP4 activities have been shown in patients with both active Crohn's disease and ulcerative colitis (6). These data show that GLP-2 has an important role in the mucosal healing of inflammatory intestinal diseases.

In a study on mice regarding an experimental colon cancer model created by GLP-2 infusion after 1,2-dimethylhydralasine (DMH), which was thought to be similar to human colon carcinogenesis (7), suggested that GLP-2 and GLP-2R expression may be an important factor in the development of CRC.

Considering the results of experimental studies and the proliferative and antiapoptotic effects of GLP-2, especially in the colon, it is thought that it plays an important role in human colon cancer and polyp development.

There are no data available that point to the effect of GLP-2 and its receptors on human CRC and colonic polyp tissue. In this study, our aim was to evaluate the role of GLP-2 and its receptor in the development of CRC pathogenesis.

MATERIALS AND METHODS

Study Group

The study included 30 patients in the colon cancer group and 20 patients in the colon polyp group, who were diagnosed by endoscopic and pathologic examination in Dokuz Eylül University, Gastroenterology Department, within a two-year period (October 2006-October 2008). Patients were studied in accordance with the protocol approved by the Institutional Committee on Human Subjects of the Dokuz Eylül Medical University. All patients gave written informed consents.

Biopsies were collected from normal-appearing colon mucosae of the same patients for the aim of comparison. Age, sex, additional illnesses, and medications of all patients were recorded.

Before colonoscopy, all patients were interviewed for any coagulopathy that could inhibit the biopsy procedure, medications affecting the parameters of coagulation and fibrinolysis (heparin, warfarin, etc.), chronic liver failure, and renal failure. Patients determined as having any of the above were excluded from the study.

Pathologic Examination

Five micron sections were taken to poly-L-lysine coated slides from each representative paraffin blocks for immunohistochemical staining. Standard streptavidin biotin immunoperoxidase method was used for immunostaining with GLP-2 receptor antibody (Genetex, dilution:1/100-1/200, 1mg/ml). The tissue sections were deparaffinized in xylene, rehydrated in alcohol series and immersed in distilled water. Antigen retrieval was performed in 0.1 mol/L Citrate buffer (pH: 5.5) in 99 C° for 20 minutes. Afterwards, Lab Vision Autostainer 360 was used for further staining. In this procedure, endogenous peroxidase activity was blocked in 0.3 % H₂O₂ for 15 minutes and then the sections were washed with Tris. The sections were then incubated in Large Volume Ultra V Block for 5 minutes and then the primary antibody (GLP-2, 1:100-1:200 dilution rate) for 60 minutes. After washing in Tris, incubation with the secondary biotinylated antibody (Thermo, LabVision) was performed. Diaminobenzidine (DAB) was used as

chromogen and the sections were counterstained with Mayer's hematoxylen for 30 seconds. Then sections were dehydrated in alcohol series, immersed in xylol for 10 minutes and were closed in DA-KO Coverslipper. Colon tissue was used as positive control. The staining profile was evaluated in light microscope (Nikon eclipse 80 i). For the evaluation of GLP-2 staining in the cases with adenocarcinoma, nuclear and cytoplasmic expression in less than 30% of tumor cells was scored as (+) and expression in more than 30% of tumor cells was scored as (++) staining.

Statistical Analysis

The data of immunohistochemical evaluation were statistically analyzed using computer software (SPSS 11.0, Chicago, IL, USA). For descriptive findings, percentage calculation, averaging and standard deviation were used.

The probability level of 0.05 or less was considered statistically significant. Chi-square test was used in analysis comparing adenocarcinoma with normal tissues and polyps with normal tissues. However, as the expected values in table cells were below 5, Fisher's exact test was used in the chi-square test, which was used for comparing polyps and adenocarcinoma for GLP-2R expression.

RESULTS

The study was carried out in a total of 30 patients (17 male, 13 female) with pathologically proven colorectal adenocarcinoma along with a total of 20 patients (12 male, 8 female) with adenoma. Cancer tissues were collected from each of the 30 patients. Polyp tissue specimens were collected from a total of 20 patients with adenoma, as follows: 1 tissue from 11 patients, 2 tissues from 8 patients and 3 tissues from 1 patient. The average age of patients with CRC was 62 ± 10 years (range: 35-85), while the average age of adenoma patients was 64 ± 9.4 years (range: 46-82). No difference was found between the CRC and adenoma groups with regards to age and sex.

In CRC, tumor localization was in the rectum in 11, sigmoid in 4, rectosigmoid in 4, and other areas of the colon in 11 patients (Table 1). In the adenoma group, 30 specimens were collected from the rectum of 9, sigmoid of 3, descending colon of 6, transverse colon of 7, ascending colon of 3, and cecum of 2 patients. Thirty adenocarcinoma cases were detected in the cancer group. Nineteen of the adenomas were tubular, 8 were tubulovillous and

3 were villous adenoma (Table 2). Eleven of the specimens in the adenoma group were 5 mm-1 cm, 4 were 1-2 cm and 15 were >2 cm in diameter.

Immunohistochemical Evaluation

GLP-2R antibody (1:100-1:200, 1 mg/ml) was studied immunohistochemically in all cases. Normal mucosa tissues neighboring the CRC and polyp were taken as controls, and staining showing GLP-2R expression in enteroendocrine cells was observed in all (Figure 1). Detection of chromogranin positivity and actin negativity in normal mucosa cells shows that these GLP-2R stained cells have enteroendocrine cell characteristics. None of the adenoma cases showed staining (Figure 2). In the cancer group, focal cytoplasmic staining was observed in 6 patients (20%) and no staining was seen in 24 patients (80%) (Figures 3, 4). One of these 6 cases was recorded as having (++) staining and 5 of them were recorded as having (+) staining.

Table 1. Demographic and clinical characteristics of colorectal cancer cases.

Age	62 ± 10.7 years
Sex	n
Male	17
Female	13
Localization	
Rectum	11
Sigmoid	4
Rectosigmoid	4
Descending colon	4
Transverse colon	2
Cecum	5

Table 2. Demographic and pathologic characteristic of adenoma patients

Age	64 ± 9.4
Sex	n
Male	12
Female	8
Localization	
Rectosigmoid	12
Descending colon	6
Transverse colon	7
Ascending colon	3
Cecum	2
Histology	
Tubular adenoma	19
Tubulovillous adenoma	8
Villi	3

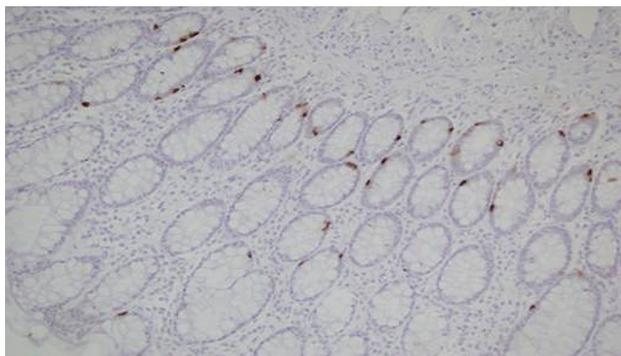


Figure 1. GLP-2R positivity in endocrine cells in normal mucosa (x 20 magnification).

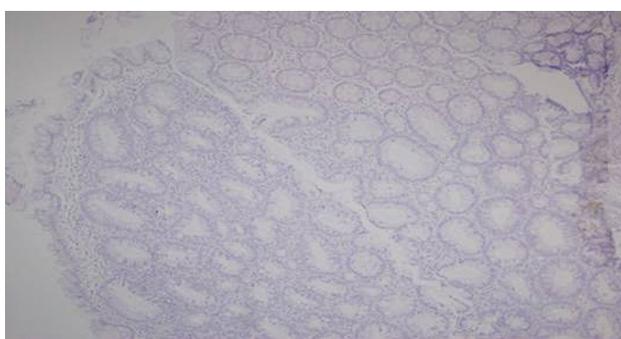


Figure 2. GLP-2R negativity in tubular adenoma cells (x 10 magnification).

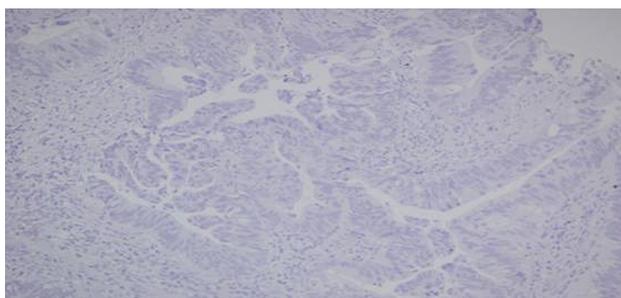


Figure 3. GLP-2R negativity in adenocarcinoma cells (x 20 magnification).

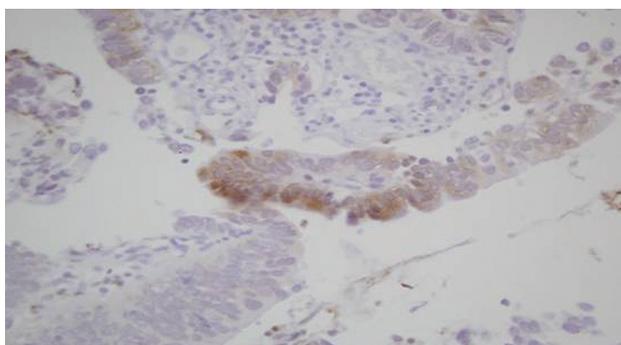


Figure 4. GLP-2R positivity in adenocarcinoma cells (x 40 magnification).

Considering diagnosis and stain relation, significant staining was observed in the adenoma group in comparison to normal mucosa tissue ($X^2 = 56$, Yates correction, $p < 0.05$) and in the cancer group in comparison to normal mucosa tissue with regards to GLP-2R expression ($X^2 = 36.74$, Yates correction, $p < 0.05$). Significant staining was observed in the cancer group in comparison to the adenoma group (Fisher's exact test-2 way, $p < 0.05$) (Table 3).

Table 3. Comparison of positive stained GLP-2R among adenocancer, adenoma and normal colon tissue

	GLP-2R staining	
	Positive stained	Negative stained
Cancer ^α	6	24
Adenoma ^β	0	30
Normal tissue ^χ	30	0
α vs β: $p = 0.023$		
β vs χ: $p = 0.000$		
α vs χ: $p = 0.000$		

DISCUSSION

Along with environmental factors, genetic factors also play an important role in the development of CRC. Cellular oncogenes, growth factors and receptors are cited as having a role in regulating the growth and development of CRC (2). Recent findings of the studies in humans and animals anticipate that growth factors have a critical role in cell transformation and tumorigenesis.

Insulin-like growth factor-1 receptor (IGF-1R), a cellular membrane receptor showing tyrosine kinase activity, was detected in the crypt-villus axis of normal human intestinal epithelium gradually decreasing distally. Knock-out studies on mice support the key role of IGF-1R and ligands in cellular growth and development. It is determined that there is an increase in proliferation in colon epithelium cells of acromegalic patients, and accordingly, CRC and adenoma risk increases. Hypoxia and some stimulants, which have not been clearly determined yet, stimulate the tumor cells, the cells causing inflammation and ligament cells, and this stimulation causes the formation of some molecules of angiogenesis effect, such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor-beta (TGF- β) and platelet-derived growth factor (PDGF).

The GLP-2R studies in humans and animals show that plasma concentration increases especially after intestinal damage. Considering the results of experimental studies and the proliferative and antiapoptotic effects of GLP-2, especially in the colon, it is thought that GLP-2 may play an important role in human colon cancer and polyp development.

The first evidences revealing the relation between increased peptides -similar to glucagon- and intestinal villous hyperplasia were the outcome studies carried out by Gleeson and co-workers (8) on patients who had small intestinal hyperplasia and tumors secreting glucagon. Jejunal biopsies of a 44-year-old female patient with renal endocrine tumor, who was studied upon complaints of vomiting, constipation and abdominal distension, showed extension in villi. The study on a specimen of 7 cm from the right kidney tissue showed glucagon immunopositivity, and the patient's serum glucagon level in that period was found to be 10 times the normal level. Weeks after the tumor was removed, small intestinal villous hyperplasia showed regression. All these findings lead us to think that peptides oscillating due to glucagon or the tumor are responsible for the changes in small intestinal mucosa.

Chance *et al.*'s (9) study on rats showed that the villous atrophy and mucosal hypoplasia in animals only fed by intravenous route regressed under the condition that simultaneous GLP-2 infusion was provided. After GLP-2 treatment was terminated, the changes in intestinal mucosa returned to the initial situation.

Similarly, in a study on mice by Boushey and colleagues, enteritis was induced by indomethacin. Mice was given Human GLP-2 analog before or simultaneously with indomethacin or after 48-72 hours and survival rate increased significantly in all of these three groups. GLP-2 treated mice exhibited reduced histological evidence of disease activity, fewer intestinal ulcerations and decreased myeloperoxidase activity in the small bowel. Decrease in mortality was connected to the fact that GLP-2 decreases the bacterial infection frequency by decreasing illness activity, intestinal cytokine oscillation and intestinal permeability considerably. GLP-2 had positive effects on ischemic intestinal damage in rats. GLP-2 given after superior mesenteric arterial blockage provided mucosal renewal and considerable decrease in mortality (11).

The protective effects of GLP-2 were shown in a study by Drucker and colleagues (12). In the study, colitis was induced with dextran sulphate in the large intestine, and illness activity and intestinal interleukin (IL) expression (especially IL-1) were decreased and there was also a significant decrease in weight loss after concomitant GLP-2 was given.

Experimental studies by Tavakkolizadeh *et al.* (13) compared two groups of rats with tumor, one of which was treated by 5-fluorouracil (5-FU) while the second group was treated by GLP-2 and 5-FU; the second group showed a decrease in intestinal damage. The rats, which were given h(Gly2 GLP-2) before irinotecan -a topoisomerase inhibitor- were compared with rats given only irinotecan, and it was seen that the first group showed considerable decrease in bacterial infection, intestinal damage and mortality. Histological and biochemical analysis presented a decrease in crypt cellular apoptosis and intestinal caspase-8 activity (14).

In a study on eight patients suffering from short bowel syndrome and related malabsorption, GLP-2 was given subcutaneously (SC) to patients twice a day for 35 days (4). At the end of the treatment, there was an increase in weight and body mass index of the patients, decrease in fat mass, and increase in bone density, and five of the patients showed an increase in villi length in the small intestine, crypt depth and mitotic index. During this period, patients adapted well to the treatment, and no abnormality was observed in either biochemical or hematological analysis. All of these positive results are promising for the use of GLP-2 for potential treatment purposes and the development of more potent resistant GLP-2 analogs.

The fact that GLP-2 has useful proliferative effects on intestinal diseases raises the question of whether this protein has a place in cancer development. In a study carried out on mice, an experimental colon cancer model was induced by giving GLP-2 after DMH and it was thought that the results would be similar to humans clinicopathologically (7).

Thulesen and colleagues (15) gave carcinogen DMH via SC route to 210 female mice once per week for 12 weeks. At the end of this period, mice were divided into three groups. One group received SC GLP-2, the second SC stabile analog Gly2-GLP-2 and the third placebo, and these groups were also divided into sub-groups of 10-day and 1-month treatment periods. Afterwards, postmor-

tem analysis was performed on the colon and rectum tissues of these mice. Increase in intestinal mass was observed in mice given GLP-2 and more significantly in mice given Gly2-GLP-2 when compared to placebo subjects. Tubular adenoma histology of all mice showed development of colon polyps. This study showed us that Gly2-GLP-2 considerably increased colon cancer incidence. However, there are no reports in the literature showing a relation between the formation of human colon cancer and its precursor colon polyps and GLP-2 and GLP-2R.

Two independent studies on tissue culture by Bulut and colleagues (16) proved by flow cytometry and immunoblotting methods that GLP-2 caused an increase in proliferation of Caco-2 and Colo-320, which are human-derived epithelium cells. Yet, there is no study on GLP-2R expression during development of colon cancer in humans. Based on the proliferative effects of GLP-2 in the small and large intestine, it is anticipated that there is a progressive increase in GLP-2R expression in transition from mucosa to adenoma, carcinoma and metastasis.

Masur et al. (17) analyzed the proliferation and migratory activities of two series of colon cancer cells after GLP-2 or GLP-2+dipeptidyl peptidase 4 (DPPIV) inhibitors incubation using flow cytometry and anti GLP-2R antibody, and then checked the results with reverse transcription-polymerase chain reaction (RT-PCR). It was found that the doubling span of cells decreases and migratory activity significantly increases when GLP-2 is given in combination with DPPIV inhibitor. The authors concluded that usage of DPPIV inhibitor may affect GLP-2 indirectly causing intestinal tumor development and that existing colon cancer cells may acquire metastasis potential.

Yusta and colleagues (18) showed the existence of GLP-2R (+) cells immunohistochemically in the human stomach and small and large intestinal epithelium. It was understood that these GLP-2(+) cells also presented chromogranin positivity. However, in that study, GLP-2R expression was detected by combination of Northern blotting, RT-PCR and immunohistochemical studies. Cells with lower levels of GLP-2R mRNA transcription could thus be detected by the more sensitive Northern blotting and RT-PCR methods.

In our study, we determined that enteroendocrine cells of all 60 tissues, which were analyzed by im-

munohistochemical method in normal colon mucosa around the adenoma or cancer tissue, expressed GLP-2R. Nevertheless, no staining reflecting GLP-2R expression was observed in adenoma cases. In carcinoma cases, we determined focal cytoplasmic staining in parallel with GLP-2R expression in only 6 cases (20%).

Considering the fact that lower levels of antigen expressions cannot be determined using immunohistochemical methods, higher levels of GLP-2R expression might have been detected in the carcinoma tissue if the more sensitive RT-PCR method had been used in harmony with the study of Yusta and colleagues (18).

In the previous studies regarding colon cancer pathogenesis on growth factors, distinctive differences were found in receptor positivity figures. The reasons for these distinctive differences may be a) tissue type (usage of fresh tissue, paraffinized tissue, and cancer culture series), b) detection type, such as enzyme immune assay and immunohistochemical study, and c) absence of standard cut-off values for receptors in the studies and varying cut-off values with each analyst. In our study, the reason that greater GLP-2R expression increase could not be detected in colon adenoma and cancer case specimens might be due not only to the lower sensitivity of the immunohistochemical study that was used as the determining method, but also because of the tissue type we studied.

In our study, no GLP-2R expression was detected in adenoma cases, but some focal cytoplasmic expression was detected in cancer cases. As we know, adenomas are pioneer lesions in colon cancer pathogenesis, and the fact that GLP-2R expression was not observed in adenoma cases but was significantly observed in cancer cases leads us to think GLP-2R expression has a role in colon cancer carcinogenesis in advanced phases. The gradual increase in GLP-2R expression in the adenoma, dysplasia and carcinoma process in colon carcinogenesis can be a sign of cellular proliferation speed.

In conclusion, GLP-2R expression in colon polyps was not detected in humans in contrast to the study on mice. This leads us to think GLP-2R expression is not a factor in adenoma-cancer pathogenesis. However, GLP-2R expression might play role in the advanced level of colon cancer and probably in metastasis pathogenesis. More studies are needed on this subject with more facts and different methods.

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