Expression of leptin and leptin receptors in colorectal cancer - an immunohistochemical study

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Obesity is demonstrated to be a risk factor in the development of cancers of various organs, such as colon, prostate, pancreas and so on. Leptine (LEP) is the most renowned of the adipokines. As a hormone, it mediates its effect through leptin receptor (LEPR), which is widely expressed in various tissues including colon mucosa. In this study, we have investigated the degree of expression of LEP and LEPR in colorectal cancer (CRC). We collected 44 surgically resected colon cancer tissues along with normal adjacent colon tissue (NACT) from a sample of CRC patients from Malaysian population and looked for leptin and leptin receptor using immunohistochemistry (IHC). All the samples showed low presence of both LEP and LEPR in NACT. While both LEP and LEPR were present at high intensity in the cancerous tissues with 100% and 97.7% prevalence respectively. Both were sparsed in the cytoplasm and were concentrated beneath the cell membrane. However, we did not find any significant correlation between their expression and pathological parameters like grade, tumor size, and lymph node involvement. Our study further emphasizes the possible causal role of LEP and LEPR with CRC, and also the prospect of using LEPR as a possible therapeutic target.
Expression of leptin and leptin receptors in colorectal cancer - an immunohistochemical study

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Abstract

Obesity is demonstrated to be a risk factor in the development of cancers of various organs, such as colon, prostate, pancreas and so on. Leptin (LEP) is the most renowned of the adipokines. As a hormone, it mediates its effect through leptin receptor (LEPR), which is widely expressed in various tissues including colon mucosa. In this study, we have investigated the degree of expression of LEP and LEPR in colorectal cancer (CRC). We collected 44 surgically resected...
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Introduction

Adiposity is considered a major health problem of pandemic dimension and involves both developed and developing countries (1–3). Much surpassing its physical and mechanical burden, it comes out as a significant metabolic player in various endocrine and cardiovascular disorders, and has also been demonstrated to be a risk factor in the development of cancers of various organs, such as colon, esophagus, gall bladder, pancreas, kidney, thyroid, prostate, uterus and breast (1,4,5).

Colorectal cancer (CRC) is one of the most common causes of cancer-related death worldwide (6). The epidemiological evidence shows a clear link between colon cancer and obesity (7–9). The two are associated with a sedentary lifestyle, high-energy diets, and limited consumption of vegetables, fruits, and fibers (7–9). The relationship between the two, if any, needs to be studied through deeper investigations.
Adipose tissue synthesizes and secretes a number of hormones and cytokines, also called adipokines, such as leptin (LEP), adiponectin, resistin, apelin, omentin, tumor necrosis factor-a, IL-6, etc. (10–12) The tissue acts upon other organs through the adipokines. The most widely researched and most significant obesity-related adipokine is leptin (LEP) (13–17).

It is established that LEP mediates its action through its receptor (LEPR) (18–20). Therefore, LEPR also has become an important target of research as part of the LEP-LEPR system for humoral control of organs and their functions.

A baffling feature of LEP is that although its most abundant source is white adipose tissue (WAT), it is also secreted by many other tissues; for example, placenta (21,22), stomach (23), mammary gland (24), brain and pituitary, (25), colon (26), ovaries (27), bone and cartilage cells (28), testicles (29), skeletal muscle (30), and so on.

Likewise, the leptin receptor (LEPR) is found in many tissues other than its canonical target organ - the hypothalamus. The receptor is expressed in placenta (31,32), gastric mucosa (33–35), lung (36), endometrium (37), immune cells (38), liver (39), and pancreas (40). Adipose tissue and gastric mucosa also produce forms of LEPR that are soluble in plasma and tissue fluids and remain bound to circulating leptin increasing its half-life (41).

Consistent with the wide occurrence of LEP and LEPR, the system besides its canonical function of balancing food intake and body mass (13,14,16–20,42,43) is found to regulate a plethora of processes and pathways, including growth of and secretion from gastric epithelial cells(33),
secretion of mucin from colonic goblet cells (44); reproduction, bone remodeling, insulin signaling, neuroendocrine function (45) inflammatory response (46,47), regulation of blood pressure, thyroid hormone release, immune factors and cells (23,48,49), pancreatic beta cells, regulation of fat and glucose metabolism, insulin sensitivity, and so on (43).

The most important action of the LEP-LEPR system in relation to tumorigenesis is its growth effects. It has been found to be required as a growth factor for mammary gland (50–52), fetal and neonatal growth (22,53), lung (36), hepatic cells (54), pancreatic \( \beta \) cell growth and function (55), colonic epithelial cells (26), and so on. Bone growth and bone mass are severely reduced in LEP deficient (ob/ob) animals, but it can be restored with the administration of LEP (56). More significantly, the system can interact with a number of other hormonal mediators including insulin, glucagon, the insulin-like growth factors, estrogen, progesterone, growth hormone and glucocorticoids (57). Notably, to execute its growth effects, it has been demonstrated that the system promotes cell proliferation, angiogenesis, mesenchymal transformation, and exerts anti-apoptotic effect (17,58–63), which also are essential requirements of tumorigenesis (14,62,64).

As evidence to the above hypothesis, LEP and LEPR have been demonstrated in unusually high concentration in various cancerous tissues by many authors (65). They are found in high concentration in breast carcinoma (66–68), leukemia (69), as well as prostate (70), esophagus (71), gastric (72), lung (73), adenocarcinomas, etc.

Many authors have reported high presence of LEP in colorectal cancerous cells (74–79). Recently, a study in Saudi Arabia on colorectal tumors has found LEP in a very high percentage (93%) of the samples on immunostaining (80). Nevertheless, some authors reported that in
advanced cancers LEP expression diminishes (72) (74), suggesting silencing of LEP expression in an advanced stage, which indicates the anti-tumorigenic role of the LEP. Again Aparicio et al. have reported that LEP acts as an in vitro growth factor for colon cancer cells, but does not promote tumor growth in vivo (81). Such conflicting reports make the case of LEP in CRC all the more interesting.

Many studies have also demonstrated over-expression of LEPR in colon cancer (26,81–85). Altered patterns of LEPR expression have been reported by a number of authors (46,86–88) It has been proposed that phenotypic variation of LEPR expression may give variants with better prognosis (46,87). Some other authors have reported that in CRC patients, tissue LEP and LEPR are related significantly to the grade of tumor differentiation, depth of bowel wall invasion, and distant metastasis (89). The confounding complexity of LEP-LEPR system in human colon cancer patients obviously demands wider studies.

In this paper, we report an investigation of the degree of expression/presence of both LEP and LEPR using immunohistochemistry in colorectal mucosa in surgically resected CRC specimens from a sample of the Malaysian population.

**Materials & Methods**

**Tissue samples:**

A total of 44 paraffin blocks of colorectal cancer (CRC) were taken from the histopathology laboratory in Hospital Tengku Ampuan Afzan (HTAA), Kuantan, Pahang, Malaysia. Colon samples were taken from resected tumors along with the adjacent normal tissue, which was used
as controls. Selection of patients and clinical diagnosis were done in collaboration with the Department of Histopathology at HTAA from January 2017 to May 2018.

The age distribution of the patients were as such: 4(9.1%), 5(11.4%), 8(18.2%), 19(43.2%), 6(13.6%) and 2(4.5%) were in their 30s, 40s, 50s, 60s, 70s, and 80s respectively. Most of the samples were from males 26(59.1%). Most 40(91%) were moderately differentiated, only 2(4.5%) of samples were well differentiated and only 2(4.5%) undifferentiated. Age, sex and tumor grade distribution of the patients are shown in three short Tables 1-3 below.

Tissue collection and preparation

The study protocol will include studying of histopathological samples from 44 patients with colorectal cancer. From the patients' forty-four pairs of histopathological samples, each pair consisting of a sample from the cancer tissue and another from adjacent normal colon tissue (ANCT) were obtained by the histopathological laboratory (HTAA). The tissue samples taken from ANCT of the same patient were considered as controls. All tissue samples were subjected to histopathological examination using immunohistochemistry procedure detailed below.

A rotatory microtome machine was used for sectioning.Trimming and sectioning were done with about 4-5μm thick. All slides were stained with H&E and stored until the histopathological examination was achieved.

All histopathological procedures were conducted at the research laboratories in the Department of Basic Medical Sciences, Faculty of Medicine, International Islamic University Malaysia.
(IIUM), Kuantan, Pahang, Malaysia in collaboration with the Department of Pathology at HTAA.

**Immunohistochemistry (IHC)**

IHC for Leptin and Leptin receptors were done using Dako Autostainer (Denmark), utilizing the REAL™ EnVision™ Detection System, Peroxidase/Dab+, Rabbit protocols from Dako, Denmark. Tissue sections were made on saline coated glass slides for IHC staining. AB9749 Abcam Rabbit polyclonal to Leptin and AB104403 Abcam Rabbit polyclonal to Leptin Receptor were used.

Anti-leptin antibody and anti-leptin receptor antibody were diluted to 1:1000 and 1:100 respectively by adding the appropriate amount of Dako antibody diluent.

IHC staining was performed using Dako Autostainer according to the manufacturer’s protocol. The IHC-stained tissue sections were counterstained in hematoxylin solution for 15 seconds and were rinsed in running tap water for 5 minutes before differentiating in 1% acid alcohol for 2 dips. The sections were then rinsed in running tap water for 5 minutes. The tissue sections were dehydrated in 70% alcohol for 3 minutes, followed by 80%, 90% and absolute alcohol 2 and 1.5 minutes respectively. Then, the tissue sections were dried in an oven for 10 minutes at 37°C, followed by clearing in xylene twice for 2 minutes. The slides were finally cover-slipped with DPX mounting medium.
IHC stained slides were examined under low and high power magnification using an Olympus BX15 (Tokyo, Japan) light microscope. The result was then analyzed for the expression of leptin and Leptin receptors.

In our study, the immunoreactivity of Leptin and leptin receptor has been examined, by two pathologists, for staining intensity and positively stained cells percentage. The frequency of positive cells was evaluated by applying a semiquantitative method. Staining intensity has been given scores 0, 1, 2, and 3 demonstrating negative, faint, moderate, and strong staining respectively. For simplification of analysis, scores of staining intensity have been grouped as negative, low (1+) and high (2+ and 3+), as was reported previously (74,80)

Statistical analysis

SPSS software (version 22.0) was used for all statistical calculations. Spearman's rho coefficient was used to determine correlations between the variables.

Ethical approvals

The full research protocol was approved by the International Islamic University Malaysia Research Ethical Committee (IREC). The approval reference number is IIUM/ 305/20/4/1/7. Since already recorded data and stored surgical tissue samples were issued no consent was thought necessary or feasible.
Results

Presence of leptin (LEP) and leptin receptor (LEPR) in the cancerous and the normal adjacent colon tissue samples:

We selected 44 out of 56 CRC tissue samples as qualitatively acceptable for the purpose of our study. Among the 44 CRC samples, moderately differentiated (M) tumors were significantly higher compared to well differentiated (W) and undifferentiated (U) samples, 40, 2 and 2 respectively (P<0.01, Chi-square value = 61.714). Among the cancerous tissues, all of the 44 (100%) samples stained strongly (2+ or 3+) for LEP and all but one or 43(97.7%) samples stained strongly for LEPR with the IHC technique. In contrast, all of the 44 normal adjacent colon tissue (NACT) took low stain for both LEP and LEPR. The result is tabulated in Table 4.

Results of the statistical analysis

The difference in the expressions of LEP and LEPR between the cancerous and NACT were very significant (P<0.01, Wilcoxon signed rank test). The statistical values are summarized in Table 5.

However, we did not find any significant correlation between their expression and pathological parameters like grade, tumor size, and lymph node involvement (Table 5).

Discussion

Definite association of obesity with various types of tumors readily drew attention towards its flag bearer hormone leptin (LEP), as it is the humoral mediator through which adipose tissue could exert a direct effect on other organs and processes. As an indispensable part of LEP action
is its receptor (LEPR). In this study we graded staining as 'negative,' 'low,' and 'high' (as elaborated in methodology) and observed presence of both LEP and LEPR in colorectal cancer (CRC) tissue samples in high intensity with high prevalence, 100% and 97.7% respectively (p<0.01) (Table 4). However, adjacent normal colon tissue (ANCT) were weakly and invariably immunostained for both LEP and LEPR (100%). In contrast to the previous report by Koda et al. (74) the distribution of stain in the ANCT was comparatively uniform too (Figure 1).

We do not know of any other report so far that has found such a high occurrence of LEP and LEPR in colonic or any other types of tumors. Numerically nearest prevalence of LEP (not LEPR) has been reported by Al-Maghrabi et al. (80), where LEP has been observed to be present in 93.5% of the CRC cases in the Western Province of Saudi Arabia, but moderate to strong staining in only 22.75% of the cases. While findings of Al-Maghrabi et al. (80) match with those of Jeong et al (79); previous reports by Koda et al. (74) from Europe, Paik et al. (75) from Korea, Liu et al (76) and Wang et al (77) from China showed further lower values (LEP was just present only in 51.2%, 73.5%, 72.1, and 71.3%, respectively). Although the presence of both LEP and LEPR in CRC tissues in this Malaysian sample of the population is strikingly high and interesting, yet it would be premature to draw any conclusion about whether the differences are due to regional, temporal or racial factors, before further independent studies.

Koda et al. (74) have reported that LEP is overexpressed in human CRCs relative to normal colorectal mucosa. While in normal mucosa it is low or undetectable, its level is higher in tissues adjacent to CRCs. But LEP concentration is the highest in moderately differentiated (G2) cancers compared to poorly differentiated (G3) cancers. Similarly, Al-Maghrabi et al. (80)
reported that larger size tumors gave a significantly higher proportion of negative immunostaining. Lower LEP occurrence has also been reported with less differentiated gastric adenocarcinomas, and the authors suggested silencing of LEP/LEPR expression in advanced stages of cancers (72). It is possible that in advanced stages of cancer strong oncogenes takes over the processes and the LEP system is overwhelmed at least in some cases. However, we did not find any negative correlation of LEP with advanced CRC. We had only two undifferentiated cases out of 44, but both the undifferentiated samples immunostained strongly for LEP. However, the number of cases of undifferentiated CRCs are statistically inadequate in our study to draw any conclusion. We did not find any significant correlation of LEP or LEPR staining with tumor grade. Our samples also failed to show any significant correlation either with lymph node involvement or invasion (Table 5). These findings match with those of Al-Maghrabi et al. (80) and Jeong et al. (79), however, they contrast with Koda et al. (74) and Liu et al (76).

Concomitant occurrence of high concentration LEP and LEPR in various cancerous cells are so far explained by co-expression of both in the same cell (67,90,91). However, an alternative explanation might be that overexpression of only LEPR is followed by binding and trapping of the equivalent amount of LEP from circulation. Such a situation would result in overstaining of both in the respective cells. This hypothesis is supported by the findings of Stachowicz et al. (88), who did not find mRNA for LEP in human CRC tissue samples. Interestingly, Erkasap et al. (84) reported over-expression of LEPR mRNA in human metastatic CRCs, but not in CRCs of local origin. On the other hand, some authors reported expression of LEP mRNA in normal colonic cells (92). These controversies demand much more detailed study of the expression of LEP and LEPR through mRNA studies instead of protein staining.
The very high presence of LEPR, as is found in our study, must be very significant as it is the receptor of LEP. Presence of only high level of circulating LEP cannot be sufficient to produce excessive growth promotion leading to cancer, it must need the receptor. The over-expression of LEPR, as we found in one of our previous studies with breast cancer (68), and by many authors with various cancers (46,67,85–87,90,93–95) must be significant in the carcinogenic influence of obesity or LEP. However, the claims by various authors about the effect of LEP-LEPR system in colon cancer are confounding, and maybe even contradictory (82,86,96–101). Some authors reported increased LEP levels in male but not in female CRC patients (96,97). Others showed that LEP expression in cancer tissue rises as carcinogenesis progresses (74,75) and that LEP expression in cancer tissue may be positively correlated with survival of colorectal cancer patients (75) or LEPR over-expression is associated with anti-tumor response and better prognosis (46); while some claim that serum LEP values are lower in patients with colon cancer (98,99), serum LEP levels decreases with the progress and aggressiveness of tumor (100); while others even fails to determine any significant difference in serum LEP between colon cancer patients and controls (86). An explanation to such contradictory report might lie in the fact that LEPR shows polymorphism and phenotypic variants (46,85–87) and it is very much possible that different variants respond differently to LEP stimulation - one variant might over-stimulate cells towards carcinogenesis, while another might be less sensitive to circulating LEP concentration or even act against the progress of the tumor.
Conclusions

Over-expression of both LEP and LEPR in CRC, along with similar findings in our previous studies with breast carcinoma and related works of other authors, as mentioned above, suggest strong association of LEP/LEPR system with carcinogenesis. Given the already established role of LEP/LEPR system in growth promotion, it may be assumed to be causally related to carcinogenesis, where its growth effect may go out of control resulting in tumorous growths or its hyperactivity helping the tumor to grow faster.

Since LEP/LEPR is over-expressed in so many processes, they could not be generally used as cancer markers; however, if specific phenotypic variants of LEPR are involved with cancers, which is yet to be studied, then such phenotypes could be used as cancer markers. Apparent contradictions about the role of LEPR may be due to phenotypic variants of LEPR, which may respond differently to LEP stimulation. It seems, while LEPR pathway has much potential to be a target for anti-cancer drug therapy, drugs might better be targeted to particular variants of LEPR. Therefore, elaborate characterizations of phenotypic variants of LEPR are essential for various reasons.

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Figure 1

Results from Immunohistochemical (IHC) staining

**Figure 1.** Immunohistochemical (IHC) staining for LEP (upper panel A-C), and LEPR (lower panel D-F): (A,D) Adjacent Normal Colon Tissue (ANCT) with low staining (intensity 1+), (B,E) Moderately differentiated CRC with high staining (intensity 2+), (C,F) Moderately differentiated CRC with high staining (intensity 3+); (all x200 magnification).
Table 1 (on next page)

Age distribution of patients with colorectal carcinoma.
Table 1: Age distribution of patients with colorectal carcinoma.

| Age groups: | 30s | 40s | 50s | 60s | 70s | 80s |
|-------------|-----|-----|-----|-----|-----|-----|
| (years)     |     |     |     |     |     |     |
| n=44        | 4   | 5   | 8   | 19  | 6   | 2   |
| (in %)      | (9.1)| (11.4)| (18.2)| (43.2)| (13.6)| (4.5)|
### Table 2 (on next page)

Sex distribution of patients with colorectal carcinoma
Table 2: Sex distribution of patients with colorectal carcinoma.

|   | Female | Male  |
|---|--------|-------|
| n=44 | 18(40.9%) | 26(59.1%) |
Table 3 (on next page)

Tumor grade distribution of the patients with colorectal carcinoma.
Table 3: Tumor grade distribution of the patients with colorectal carcinoma.

| Tumor Grade | Well Differentiated | Moderately Differentiated | Undifferentiated |
|-------------|---------------------|----------------------------|-----------------|
| n=44        | 2(4.5%)             | 40(91%)                    | 2(4.5%)         |
Table 4 (on next page)

Percentage of positive (low and high) cases of LEP and LEPR in IHC stained of colorectal tissue
Table 4. Percentage of positive (low and high) cases of LEP and LEPR in IHC stained of colorectal tissue

|                      | Immunoreactivity for LEP | Immunoreactivity for LEPR |
|----------------------|--------------------------|----------------------------|
|                      | low | high   | low  | high     |
| Cancer Tissue        | 0   | 44 (100%) | 1 (2.3%) | 43 (97.7%) |
| N=44                 |     |         |      |          |
| Adjacent Normal Colon tissue | 44 (100%) | 0     | 44 (100%) | 0         |
| N=44                 |     |         |      |          |
| P value*             | <0.01 | <0.01 |

*P values for significant difference in expression of LEP and LEPR between NACT and cancerous colon tissue were calculated by applying Wilcoxon signed rank test. (IHC: immunohistochemistry, LEP: leptin, LEPR: leptin receptor, NACT: normal adjacent colon tissues)
Table 5 (on next page)

Results of the statistical analysis of the correlation of LEP and LEPR with the common pathological parameters
Table 5: Results of the statistical analysis of the correlation of LEP and LEPR with the common pathological parameters.

|                     | Tumor size | Lymph node involvement | Grade (W,M,U)* |
|---------------------|------------|------------------------|----------------|
| LEP correlation coefficient | -0.054, P=0.73 | -0.205, P=0.181 | -0.000, P=1.00 |
| (Spearman's rho, P value) |            |                        |                |
| LEPR correlation coefficient | -0.018, P=0.907 | -0.206, P=0.179 | -0.285, P=0.06 |
| (Spearman's rho, P value) |            |                        |                |

*W: Well differentiated cells; M: Moderately differentiated cells; U: Undifferentiated cells.

(LEP: leptin, LEPR: leptin receptor)