Significance of Gene Amplification and Expression of HER2/neu in Colon Carcinoma

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Abstract

Introduction: Colorectal cancer is a major worldwide health problem with an annual incidence of 1.2 million and an annual mortality of over 600,000 people. Her2/neu oncogen is one of four epidermal growth factor receptors. It's located on chromosome 17q21 and encodes a 185 transmembrane protein with tyrosine kinase activity that functions as a growth factor receptor. Clinically, amplification and/or overexpression of Her2/neu has been associated with poor prognosis in a number of tumor types such as breast and ovarian cancer. Conflicting data exist about the prevalence of HER-2/neu overexpression in colorectal cancer ranging from 0 to 83%.

Aim of the Work: The aim of this study were to compare qRT-PCR and immunohistochemical expression of HER-2/neu oncogene in colorectal carcinoma and their correlation to other clinicopathological parameters.

Material and Methods: This work involved 50 cases of surgically resected colorectal cancers. HER2/neu immunohistochemistry was performed using the C-erbB-2 gene product.

Results: Out of 50 studied cases, 40 cases (80%) were HER-2/neu negative (scor 0 and 1+) and 10 cases (20%) were positive (scor 2+ and 3+). No statistically significant correlation were found between IHC expression HER2 and clinicopathological parameters. Among the examined 50 cases, 44 cases (88%) were over expression of HER-2/neu by qRT-PCR (>2) and 6 cases (12%) were under expression of HER-2/neu by qRT-PCR (<2). A significant correlation were found between expression of HER-2/neu by qRT-PCR HER2 and clinicopathological parameters and no correlation with IHC expression HER2.

Conclusion: Q-RT-PCR is now considered the gold standard for mRNA quantitative evaluation, and its application to HER2 status evaluation could contribute to method standardization and reduce reports of variability.

Key Words: Her2/neu – Colorectal carcinoma – Immunohistochemistry (IHC) – Q-RT-PCR.

Introduction

COLON cancer is the third most commonly diagnosed cancer in males and the second in females, with over 1.2 million new cases and 608,700 deaths occurred in 2008. Rates are substantially higher in males than in females [1]. In Egypt, colorectal cancer constitutes 4.2% and come at seventh rank (7th in men and 4th in women) [2].

There is strong evidence that virtually all CRCs originate from a precursor benign polyp, which makes this cancer potentially preventable by appropriate screening colonoscopy programs in patients at increased risk [2]. The current two most important and more understood pathways for the genetic pathogenesis of CRC, namely the conventional adenomatous pathway and the serrated pathway [3].

There is a genetic contribution to colorectal cancer evident by increased its incidence among persons with a family history of colorectal cancer and families in which multiple family members are affected indicating autosomal dominant inheritance of cancer susceptibility [4].

Several genes associated with colorectal cancer risk have been identified, almost all gene mutations known to cause a predisposition to colorectal cancer are inherited in an autosomal dominant fashion [8].

Genetic factors appear to influence the age at onset of colorectal cancer, people who have a first degree relative with colorectal cancer are estimated to have an average onset of this cancer about 10 years earlier than people with sporadic disease [6].

Her2/neu oncogen is one of four epidermal growth factor receptors. It's located on chromosome 17q21 and encodes a 185 transmembrane protein with tyrosine kinase activity that functions as a growth factor receptor [7]. It’s expressed in a variety of tissues of epithelial origin and plays a fundamental role in cellular proliferation and differentiation during fetal development. In adult, this gene is present as a single copy in normal cells; however, amplification of the gene and the resultant protein overexpression is seen in various malignancies such as breast and ovarian cancers [8].
pression of Her2/neu has been suggested as a factor of poor prognosis, decreased survival and increased metastasis in various malignant tumors [9].

Regarding colorectal cancer, some authors have reported an association between the occurrence and progression of this type of malignancy and Her2/neu overexpression, whereas others didn't find such correlation [10].

In clinical usage, the success of HER2/neu directed therapy in cancer breast has lead to evaluation of protein expression and gene amplification in other tumor types [11].

HER2/neu is important as a target of the monoclonal Trastuzumab. Trastuzumab is effective only in cancers where the HER2/neu receptor is overexpressed. One of the mechanisms of how trastuzumab works is that it binds to HER2/neu and increases p27, a protein that halts cell proliferation [12]. Overexpression of the HER2 gene can be suppressed by the amplification of other genes besides the use of the drug Herceptin. Research is currently being conducted to discover which disregulated genes may have this desired effect. Another monoclonal antibody, Pertuzumab, which inhibits dimerization of HER2 and HER3 receptors, is in advanced clinical trials [13].

Aim of the work:

This study aimed to compare qRT-PCR and immunohistochemical expression of HER-2/neu oncogene in colorectal carcinoma cases and its correlation to other clinicopathological criteria and prognostic factors.

Material and Methods

This is a retrospective study performed on 50 cases of colorectal carcinoma. 10 cases of non-neoplastic apparently normal colorectal tissue adjacent to selected lesions were taken as control paraffin blocks were collected from the Pathology Departments, Faculty of Medicine, Benha University and National Cancer Institute, Cairo University during the period from October 2012 till November 2013.

In this study, cases were eligible to be included if they were diagnosed as invasive colorectal carcinoma according to WHO classification of tumors of the colon and rectum [14] and undergone colectomy operations to provide adequate histologic sections for proper application of immunohistochemical marker HER2/neu. Tumor grading was done according to WHO (2010) [14]. Tumor staging was done according to TNM staging systems [15], lymphvascular invasion, marginal inflammatory reaction [16] and tumor budding [17] were also microscopically observed.

Other clinicopathological data were collected from the pathology sheet including age, sex, site, tumor size, presence of distant metastasis and clinical outcome.

Tissue preparation for histopathologic examination:

All specimens were formalin fixed in neutral formalin 10% and embedded in paraffin. Serial sections of 4 micron thickness were prepared from each tissue block, one of them was stained by Haematoxylin and eosin for histopathological re-evaluation.

Immunohistochemical staining:

For immunohistochemical staining by HER2/neu, the formalin-fixed, paraffin wax-embedded tissues were immunostained for HER2/neu, using standard methods. Monoclonal antibody of C-erbB-2 gene product was used (clone TAB250) supplied from the manufacturer. Positive reactions were visualized with diaminobenzidine, followed by counterstaining with hematoxylin. HER2/neu immunostaining results were estimated according to HER2/neu scoring system used to evaluate Hercep Test [15] (Table 1).

DNA extraction and quantitative PCR:

Quantitation of Her-2 by real time PCR:

1- RNA extraction from Formalin Fixed Paraffin Embedded tissue sections (FFPE):

- Principle and procedure:

The RNeasy FFPE procedure uses well-established RNeasy technology for RNA purification. Specially optimized lysis conditions allow total RNA to be effectively purified from FFPE tissue sections. The DNase digestion step efficiently removes DNA contamination, including highly fragmented molecules. Firstly, all paraffin is re-
moved from freshly cut (FFPE) tissue sections by treating with deparaffinization solution or using an alternative deparaffinization method. Next, samples are incubated in an optimized lysis buffer, which contains proteinase K, to release RNA from the sections.

A short incubation at a higher temperature partially reverses formalin crosslinking of the released nucleic acids, improving RNA yield and quality as well as RNA performance in downstream enzymatic assays. This is followed by a DNase treatment that is optimized to eliminate all genomic DNA, including very small DNA fragments that are often present in FFPE samples after prolonged formalin fixation and/or long storage times. Next, the lysate is mixed with Buffer RBC. Ethanol is added to provide appropriate binding conditions for RNA, and the sample is then applied to an RNeasy MinElute spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away.

- Preparation of buffers:

  A- Preparing DNase I stock solution: The lyophilized DNase I (1500 units) was dissolved in 550 µl DNase-free water.

  B- Preparing Buffer RPE: 4 volumes (44ml) ethanol (96-100%) was added to the bottle containing 11ml Buffer RPE concentrate.

- Procedure”:

  Using a scalpel, excess paraffin was removed off the sample block, sections were cut 5-20 µm thick, immediately the sections were placed in a 1.5ml microcentrifuge tube and the lid was closed, 160 µl deparaffinization solution was added, vortexed vigorously for 10s, and centrifuged to bring the sample to the bottom of the tube, incubation was done at 56ºC for 3min, and then allowed to cool at room temperature, 150 µl Buffer PKD was added, and mixed by vortexing, centrifugation was done for 1min at 11,000 x g, 10 µl proteinase K was added to the lower, clear phase. Mixed gently by pipetting up and down, Incubation was done at 56ºC for 15 minute, then at 80ºC for 15min.

  Transfer the lower, uncolored phase into a new 2ml microcentrifuge tube, incubation on ice was done for 3 minute, centrifugation was done for 5min at 20,000 x g, the supernatant was transferred to a new microcentrifuge tube without disturbance of the pellet, DNase Booster Buffer equivalent to a tenth of the total sample volume (approximately 16 µl) and 10 µl DNase I stock solution were added, mixed by inverting the tube. Centrifugation was done to collect residual liquid from the sides of the tube.

  Incubation was done at room temperature for 15min, 320 µl Buffer RBC was added to adjust binding conditions, and the lysate was mixed thoroughly, 720 µl ethanol (100%) was added to the sample, and mixed well by pipetting, 700 µl of the sample was transferred, including any precipitate that may have formed, to an RNeasy MinElute spin column placed in a 2ml collection tube (supplied). The lid was closed gently, and centrifuged for 15s at ≥8000 x g, step was repeated until the entire sample has passed through the RNeasy MinElute spin column, 500 µl Buffer RPE was used to the RNeasy MinElute spin column. The lid was closed gently, and centrifuged for 15s at ≥8000 x g, 500 µl Buffer RPE was added to the RNeasy MinElute spin column. The lid was closed gently, and centrifuged for 2min at ≥8000 x g to wash the spin column membrane. The collection tube with the flow-through was discarded.

  The RNeasy MinElute spin column was placed in a new 2ml collection tube (supplied). The lid of the spin column was opened, and centrifuged at full speed for 5min. The collection tube was discarded with the flow-through. The RNeasy MinElute spin column was placed in a new 1.5ml collection tube (supplied). 14-30 µl RNase-free water was added directly to the spin column membrane. The lid was closed gently, and centrifuged for 1min at full speed to elute the RNA. The RNA purity was assessed by The RNA concentration was quantified by NanoDrop ND-1000 (Nanodrop, USA).

2- Reverse transcription:

  Reverse transcription for the extracted RNA was done using SuperScriptII reverse transcriptase (Invitrogen Life Technologies Inc., Carlsbad, CA). The reaction was carried out for 60min at 42ºC and the reaction mixture was subsequently inactivated for 15min at 70ºC as. The cDNA was stored at –70ºC.

3- Primers probes kits:

  Primers and TaqMan probes for HER-2 and the GAPDH control reference gene were designed and synthesized according to Taqman Gene Expression Assay (assays Hs00170433-m1 and 4326317E, respectively) (Applied Biosystems, Foster City, CA, USA).

4- PCR for Her-2 mRNA:

  PCR reactions were carried out in a total volume of 20 µl according the manufacturer’s instructions. It is as follows:

  - 10ul master mix (2X), 1.25ul primer probe, 1.25ul GAPDH, 5ul cDNA and 2.5 ul H₂O.
The reaction was carried out using real time PCR for 45 cycles of: 5°C for 15 sec and 60°C for 30sec. The Relative Quantification (RQ) was given by the ratio between the mean value of the target gene and the mean value of the reference gene (GAPDH) in each sample. The relative amount of PCR product generated from each primer set was determined on the basis of the Cycle Threshold (Ct) value. The RQ was calculated by $2^{-\Delta\Delta Ct}$. HER-2 relative expression level was compared with the ratio of healthy controls. Overexpression was defined as the mean HER-2/reference gene ratio RQ >2.00.

**Results**

This study is a retrospective cross section study, conducted on fifty colorectal cancer cases collected from colectomy specimens sent the Pathology Departments, Faculty of Medicine, Benha University and National Cancer Institute, Cairo University. (42/50) cases (84%) were conventional adenocarcinomas and 8 cases (16%) were mucoid adenocarcinomas. Ages of patients ranged between 20-81 years (mean 49 ± 11.0 years). Most of the studied cases of CRC were in patients >40 years (88%). 48% of the studied cases were males and 52% were females.

The commonest location was in the right hemicolon (36%). The percentage in left hemicolon, transverse colon and rectum was (32%), (4%) and (28%) respectively. Most of the studied cases were ≤5cm in diameter (52%).

As regards to the degree of differentiation, most cases (84%) were moderately differentiated (grade II).

Regarding the depth of tumor invasion, 8 cases (16%) were T2, 38 cases (76%) were T3 and 4 cases (8%) were T4. 60% of cases were associated with metastatic nodal deposits.

It was found that, 56% of cases were associated with lymphvascular invasion, (36%) were associated with high grade tumor budding, 92% were positive for marginal inflammatory reaction and 66% of cases showed disease-free survival for 5 years. The clinical and Histopathologic characteristics of CRC cases are tabulated in (Table 2).

### Table (2): Clinical and histopathologic characteristics in studied CRC cases.

<table>
<thead>
<tr>
<th>Variable</th>
<th>No.of cases (N=50) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age</td>
<td>49 year</td>
</tr>
<tr>
<td>Gender:</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>24 (48%)</td>
</tr>
<tr>
<td>Female</td>
<td>26 (52%)</td>
</tr>
<tr>
<td>Site:</td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>36 (72%)</td>
</tr>
<tr>
<td>Rectum</td>
<td>14 (28%)</td>
</tr>
<tr>
<td>Size:</td>
<td></td>
</tr>
<tr>
<td>≤5cm</td>
<td>26 (52%)</td>
</tr>
<tr>
<td>&gt;5cm</td>
<td>24 (48%)</td>
</tr>
<tr>
<td>Histologic grade:</td>
<td></td>
</tr>
<tr>
<td>=</td>
<td>42 (84%)</td>
</tr>
<tr>
<td>≠</td>
<td>8 (16%)</td>
</tr>
<tr>
<td>Depth of invasion:</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>8 (16%)</td>
</tr>
<tr>
<td>T2</td>
<td>38 (76%)</td>
</tr>
<tr>
<td>T3</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>Lymph node involvement:</td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>20 (40%)</td>
</tr>
<tr>
<td>N1</td>
<td>18 (36%)</td>
</tr>
<tr>
<td>N2</td>
<td>12 (24%)</td>
</tr>
<tr>
<td>TNM stage:</td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>Stage II</td>
<td>12 (24%)</td>
</tr>
<tr>
<td>Stage III</td>
<td>26 (52%)</td>
</tr>
<tr>
<td>Stage IV</td>
<td>8 (16%)</td>
</tr>
<tr>
<td>Vascular invasion:</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>28 (56%)</td>
</tr>
<tr>
<td>Absent</td>
<td>22 (44%)</td>
</tr>
<tr>
<td>Tumor budding:</td>
<td></td>
</tr>
<tr>
<td>Low grade</td>
<td>32 (64%)</td>
</tr>
<tr>
<td>High grade</td>
<td>18 (32%)</td>
</tr>
<tr>
<td>Marginal inflammatory reaction:</td>
<td></td>
</tr>
<tr>
<td>Score 0</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>Score 1</td>
<td>16 (32%)</td>
</tr>
<tr>
<td>Score 2</td>
<td>20 (40%)</td>
</tr>
<tr>
<td>Score 3</td>
<td>10 (20%)</td>
</tr>
<tr>
<td>Prognosis:</td>
<td></td>
</tr>
<tr>
<td>Five-year disease-free survival</td>
<td>33 (66%)</td>
</tr>
<tr>
<td>Recurrence</td>
<td>6 (12%)</td>
</tr>
<tr>
<td>Death</td>
<td>11 (22%)</td>
</tr>
</tbody>
</table>

**Immunohistochemical results:**

Regarding immunohistochemical expression of Her-2/neu, scores (0 and +1) were considered negative, while scores (+2 and +3) were considered positive. Examined control cases showed positive expression of Her-2/neu in 100% of cases.

Most of the studied cases (40/50) (80%) were considered negative (Table 3).

In spite of the fact that no significant correlation was detected between scoring of Her-2/neu expression in tumor cells and histological types ($p$-value >0.05), however, all cases of mucoid adenocarcinoma were Her-2/neu negative (Table 5).

No statistically significant correlation was found between IHC expression of HER2/neu in tumor cells and tumor site, size, histologic grade, depth of invasion, lymph node metastasis and TNM stage ($p$-value >0.05) (Table 5).

No statistically significant correlation was found between IHC expression of HER2/neu in tumor
cells and lymphovascular invasion, tumor budding, and marginal inflammatory reaction (<p-value>0.05) (Table 5).

As regard the correlation between IHC expression of HER2/neu in tumor cells and clinical outcome, Log-rank test showed no significant correlations between IHC expression of HER2/neu and survival (<p-value>0.05) (Table 5).

Quantitative real time PCR results:

Examined control cases showed under expression (2) in all control cases (100%) among the examined 50 cases of colorectal carcinoma, 44 cases (88%) showed over expression (>2) while 6 cases (12%) showed under expression (<2) (Table 4).

There was a positive statistical correlation between HER2/neu expression by qRT-PCR in tumor cells and tumor grade, depth of invasion, LN metastasis, TNM stage, lymphovascular invasion, tumor budding and survival rate of CRC (<p-value>0.05) (Table 5).

No statistically significant correlation was found between IHC expression of HER2/neu in tumor cells and tumor site, marginal inflammatory reaction (<p-value>0.05) (Table 5).

Table (3): Scoring of HER2/neu in the studied CRC cases.

<table>
<thead>
<tr>
<th>Scoring of HER2/neu</th>
<th>Frequency</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>1+</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>2+</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>3+</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>

Table (4): Transcript expression by qRT-PCR.

<table>
<thead>
<tr>
<th>HER-2/neu ratio</th>
<th>Frequency</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Over expression &gt;2</td>
<td>44</td>
<td>88</td>
</tr>
<tr>
<td>Under expression ≤2</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>

Table (5): Correlation of clinicopathological data and Her2/neu expression by IHC and RT-qPCR.

<table>
<thead>
<tr>
<th>HER-2/neu expression</th>
<th>0</th>
<th>1+</th>
<th>2+</th>
<th>3+</th>
<th>Ratio 2.00</th>
<th>Ratio &gt;2.00</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score 0</td>
<td>6</td>
<td>26</td>
<td>8</td>
<td>2</td>
<td>(14.2%)</td>
<td>(19.4%)</td>
<td>50</td>
</tr>
<tr>
<td>Score 1</td>
<td>2</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>(22.5%)</td>
<td>(17%)</td>
<td>8</td>
</tr>
<tr>
<td>Score 2</td>
<td>4</td>
<td>22</td>
<td>8</td>
<td>0</td>
<td>(28.5%)</td>
<td>(22.2%)</td>
<td>32</td>
</tr>
<tr>
<td>Score 3</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>(15.6%)</td>
<td>(11.1%)</td>
<td>20</td>
</tr>
<tr>
<td>Tumor budding:</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>(15.6%)</td>
<td>(21.1%)</td>
<td>12</td>
</tr>
<tr>
<td>Absent:</td>
<td>2</td>
<td>10</td>
<td>6</td>
<td>2</td>
<td>(10%)</td>
<td>(30%)</td>
<td>18</td>
</tr>
<tr>
<td>Present:</td>
<td>4</td>
<td>22</td>
<td>2</td>
<td>0</td>
<td>(22.2%)</td>
<td>(7.7%)</td>
<td>28</td>
</tr>
<tr>
<td>TNM stage:</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>(100%)</td>
<td>(100%)</td>
<td>10</td>
</tr>
<tr>
<td>Stage I</td>
<td>0</td>
<td>6</td>
<td>4</td>
<td>0</td>
<td>(50%)</td>
<td>(33.3%)</td>
<td>10</td>
</tr>
<tr>
<td>Stage II</td>
<td>4</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>(15.4%)</td>
<td>(76.9%)</td>
<td>4</td>
</tr>
<tr>
<td>Stage III</td>
<td>5</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>(50%)</td>
<td>(76.9%)</td>
<td>10</td>
</tr>
<tr>
<td>Stage IV</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>(25%)</td>
<td>(25%)</td>
<td>4</td>
</tr>
<tr>
<td>Lymph-vascular invasion:</td>
<td>0</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>(21.4%)</td>
<td>(14.3%)</td>
<td>12</td>
</tr>
<tr>
<td>Present:</td>
<td>2</td>
<td>14</td>
<td>4</td>
<td>2</td>
<td>(9.1%)</td>
<td>(18.2%)</td>
<td>8</td>
</tr>
<tr>
<td>Absent:</td>
<td>4</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>(25.6%)</td>
<td>(18.2%)</td>
<td>16</td>
</tr>
<tr>
<td>High grade:</td>
<td>4</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>(22.2%)</td>
<td>(18.2%)</td>
<td>4</td>
</tr>
<tr>
<td>Tumor budding:</td>
<td>4</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>(22.2%)</td>
<td>(18.2%)</td>
<td>4</td>
</tr>
<tr>
<td>Low grade:</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>(0%)</td>
<td>(0%)</td>
<td>0</td>
</tr>
<tr>
<td>Marginal inflammatory:</td>
<td>4</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>(22.2%)</td>
<td>(18.2%)</td>
<td>4</td>
</tr>
<tr>
<td>Score 0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>(0%)</td>
<td>(0%)</td>
<td>0</td>
</tr>
<tr>
<td>Score 1</td>
<td>4</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>(25%)</td>
<td>(16.7%)</td>
<td>12</td>
</tr>
<tr>
<td>Score 2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>(0%)</td>
<td>(0%)</td>
<td>0</td>
</tr>
<tr>
<td>Score 3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>(0%)</td>
<td>(0%)</td>
<td>0</td>
</tr>
<tr>
<td>Prognosis:</td>
<td>5</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>(3.1%)</td>
<td>(1.9%)</td>
<td>3</td>
</tr>
<tr>
<td>5-year disease-free survival:</td>
<td>0</td>
<td>29</td>
<td>3</td>
<td>1</td>
<td>(87.6%)</td>
<td>(93.3%)</td>
<td>30</td>
</tr>
<tr>
<td>Recurrence:</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>(50%)</td>
<td>(50%)</td>
<td>3</td>
</tr>
<tr>
<td>Death:</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>(36.4%)</td>
<td>(18.1%)</td>
<td>7</td>
</tr>
<tr>
<td>qRT-PCR:</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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Significance of Gene Amplification & Expression of HER2/neu in Colon Carcinoma

Fig. (A): Colorectal conventional adenocarcinoma with vascular invasion (H & E X400). (B): Conventional adenocarcinoma with marginal inflammatory reaction score 3 (H & E X100). (C): Colorectal conventional adenocarcinoma showing low grade tumor budding, vascular invasion and marginal inflammatory reaction score 0 (H & E X100). (D): Colorectal conventional adenocarcinoma with high grade tumor budding (H & E X100).

Fig. (E): Adenocarcinoma showed negative expression for HER-2/neu, score 0 (streptavidin/biotin DAB X100). (F): Adenocarcinoma showed negative expression for HER-2/neu, score 1+ (streptavidin/biotin DAB X100). (G): Adenocarcinoma showed moderate membranous positive expression for HER-2/neu, score 2+ (streptavidin/biotin DAB X100). (H): Adenocarcinoma showed strong membranous positive expression for HER-2/neu score 3+ (streptavidin/biotin DAB X100).
Discussion

Colorectal cancer is a major worldwide health problem with an annual incidence of 1.2 million and an annual mortality of over 600,000 people [18].

In Egypt, colorectal cancer constitutes 4.2% and come at seventh rank (7th in men and 4th in women) and in United States it constitutes 10.6% of male cancers and 10.8 of female cancers, affecting one person in twenty during life time with an age adjusted incidence rate of 54 per 100,000 in males and 40 per 100,000 among females [2]. In united states CRC is the third leading cause of cancer deaths in both males and females [1].

There is strong evidence that virtually all CRCs originate from a precursor benign polyp, which makes this cancer potentially preventable by appropriate screening colonoscopy programs in patients at increased risk [2]. The current two most important and more understood pathways for the genetic pathogenesis of CRC, namely the conventional adenomatous pathway and the serrated pathway [3].

HER-2/neu which is one of Epidermal Growth Factor Receptors (EGFR), is one of the most important genes along the colorectal carcinogenesis pathway. EGFR is a transmembran tyrosine kinase receptor composed of extra cellular ligand binding domain, a short transmembran domain, and a cytoplasmic protein tyrosine kinase domain [7].

Overexpression of EGFR has been suggested as a factor of poor prognosis, decreased survival and increased metastasis in various malignant tumors including colorectal cancer [9]. HER-2/neu expression has been reported in CRC and is one of the most promising targeted therapies in treatment of this type of malignancy [19].

In the current study, this work was planned to evaluate the expression of HER-2/neu in fifty cases of colorectal carcinoma by immunohistochemical staining technique and its correlation with quantitative real time PCR analysis.

In the present study, out of 50 cases of CRC, 84% were conventional adenocarcinoma and 16% were mucoid carcinoma. These figures were similar to what was reported that most colorectal carcinomas (85%) were conventional adeno-carcinomas and 15% were classified as mucoid carcinoma [20].

In the present study, 48% of the studied cases were males and 52% were females, this is similar to what was reported that female represented the higher incidence (63%) of studied cases [21], and differs from the figure reported that female represented lower incidence 44% of cases. We could not establish the reason for the male predominance [22].

Ages of patients ranged from 20 to 80 years with mean age 49±11.0 years. Nearly similar results were obtained by Schuell et al., [22] who reported that their patient’s age ranged from 22-81 years with mean age 50.5±15.9. Moreover, 88% of them were >40 years; these figures were close to those reported by Terzi et al., [24] and Office for National Statistics, [25], where 86% of cases arising in people who are >40 years and differ from those reported by Soliman et al., [26], where 66% of cases arising in people who are <40 years. The differences between the current study and other studies may be due to the relative small sample size.

The majority of lesions in the present study were located in the colon representing 72% of cases (36% of cases were located in the right colon, 4% in the transverse colon and 32% in the left colon). The rectum came in the second place representing 28%. This means that tumors of the right colon outnumber those of the left colon. This agrees with Smyrk, [27], who stated a shift toward right-sided cancers has occurred during the second half of the twentieth century and Fenoglio-Preiser et al., [28], who stated that in low-risk countries, carcinomas of the cecum and ascending colon occur more frequently than carcinomas of the left colon, whereas in high-risk countries, colorectal carcinomas more commonly arise in the rectosigmoid region.

Regarding the histological grade of differentiation of CRC, the present study showed that moderately differentiated carcinoma was more encountered than others, representing 84% of cases, which were in accordance with the results obtained by Triest et al., [29] that 72% were moderately differentiated carcinoma. While Sharifi et al., [30], found that the commonest grade was well differentiated carcinoma representing 48.4%. This difference can be explained by the relative small sample size.

In the current study, lymph node and distant metastases at the time of diagnosis were recorded in 60% and 16% of cases respectively and according to depth of invasion, most cases (76%) were T3. Nearly similar results were obtained by Yawe et al., [31] who reported that 53% of cases with CRC positive lymph node metastasis, 13.5% of cases with distant metastases and most cases (68.4%) presented with T3. In contrast to these results
Lokeshwar and Selzer, [32] reported that 20% of patients with CRC presented with positive lymph node metastasis, 3% of cases with distant metastases and most cases (63%) presented with T2. These differences are peculiar to our patients and mainly due to the late presentation where the disease became more aggressive and advanced. Also, the low economic status are other factor for this difference.

In the current study, 56% of cases were presented with positive lymphovascular invasion and 96% of cases were associated with marginal inflammatory reaction, these figures were close to those reported by Beaton et al., [33] and Marchetti et al., [35] as they reported that 52.8% of cases were associated with positive lympho-vascular invasion of CRC and 85% of cases were associated with marginal inflammatory reaction. In contrast to these results, Marchetti et al., [35] reported that 21.5% of cases were presented with positive lymphovascular invasion and 58% of cases with CRC were associated with positive marginal inflammatory reaction. This difference can be explained by the relative small sample size.

According to TNM staging, 68% of cases in this series presented with advanced stages (III and IV). Nearly similar results were obtained by Sule et al., [36], as they reported that, 63% of cases with CRC presented with advanced stages. These results were not in agreement with the results obtained by Sis et al., [37] where the highest incidence was in stage II (46.4%). This can be attributed mainly to the absence of specific screening measures for colorectal cancer. There is considerable evidence that screening of asymptomatic persons who are at average risk can detect cancers at an early and curable stage, resulting in a reduction in mortality Walsh et al., [38]. Furthermore, some screening tests may also detect cancer-precursor lesions, which, if removed, may result in a reduced incidence of colorectal cancer Winawer et al., [39].

As regard the tumor budding, 36% of cases were associated with high grade tumor budding. These results were in agreement with Rogers et al., [40] who reported that most cases (69.5%) were associated with low grade tumor budding. In contrast to these results, Diez et al., [41] reported that most cases (75%) of CRC were associated with high grade tumor budding. The differences between the current study and other studies may be due to the relative small sample size.

As regard the clinical outcome 66% of cases showed disease-free survival for 5 years and 12% of cases were recurred. The overall mortality rate in the present study was 22% of cases, in contrast to these results, Datubo et al., [42] reported that, mortality rate was 6.1% of cases. The high mortality rate in the current study may be attributed to the fact that most patients presented late with advanced stage. This difference can be explained by the relative small sample size and the absence of specific screening measures for colorectal cancer.

Regarding the IHC staining for HER-2, in the current study 20% of the studied cases showed positive expression for HER-2/neu. These figures are close to those reported by Qingguo et al., [43], that 15.5% of the studied cases were positive. But there were a big variation between different studies; McKay et al., [44], studied the HER-2/neu protein expression in a large cohort of colorectal tumors, HER-2 was expressed in 81.8% of tumors. Other studies have described lower frequencies of HER-2 expression. Nathanson et al., [45], found HER-2 expression in only 3.6% of American patients, AL-Kuraya et al., [46], found amplification in 0% of 98 Saudi patients, Marx et al., [47] reported overexpression in 2.7% of German patients. Ross et al., [48], studied the expression of HER-2 in tumors of gastrointestinal tract. They found wide range of HER-2 expression in esophagus, gastric and colonic cancer. They concluded that either HER-2 protein overexpression or gene amplification is associated with 25% of all gastrointestinal malignancies.

Another study done by Coyatapia et al., [49], they studied the expression of HER-2 in 5751 cases of different tumor categories. Under fully standardized condition, this allowed to give reliable estimate of HER-2 overexpression across most human tumors entities. Breast cancers were among the most frequently HER-2 positive tumors. While none of 41 colon cancer analyzed in this study was HER-2 positive, this can be explained by relatively low number of cases, this rather low number raise the possibility that over expression may have been missed.

The variability of this data is not likely explained by differences in patient selection [50], technical variability in the performance of IHC staining is the most likely explanation. It is widely appreciated that IHC analysis is vulnerable to differences in tissue fixation, processing, in choice of primary antibody, detection system, epitope retrieval, interpretation and reporting [51]. The wide range of patients and gene over expression in CRC is largely due to the lack of standardization of the detection methods [52].
As regard the relation between tumor site and IHC expression of HER-2, out of 36 cases were located in colon, 72.2% of cases were negative IHC expression of HER-2 and all cases (100%) located in rectum were negative IHC expression of HER-2. No statistically significant correlation was reported (\(p > 0.05\)). The same finding was reported by Koenders et al., [53] in which they found that no statistically significant correlation was observed reported that on their work on 75 cases of CRC. In contrast a trend towards a decreasing frequency of positive HER-2 tumors from colon to rectum was reported by Gruenberger et al., [54], they study the expression of HER-2 on 675 cases of CRC. This difference can be explained by the relative small sample size.

As regard the correlation between IHC expression of HER-2 in tumor cells and histologic grade, out of 42 cases of adenocarcinoma, 76.2% of cases were negative for Her-2/neu expression while 100% of cases of mucoid carcinoma were negative for IHC expression of Her-2/neu. No statistically significant correlation were detected (\(p > 0.05\)). This results agreed with study carried by Kavanagh et al., [55] in which they found that 95.7% of cases of mucoid carcinoma were negatively stained.

As regard the correlation between IHC expression of HER-2 in tumor cells and histologic grade, most cases (85.7%) with grade (II) showed negative IHC expression of HER-2 and 50% of cases with grade (III) showed positive IHC expression of HER-2. No statistically significant correlation was detected (\(p > 0.05\)). This was in agreement with study carried by Schuell et al., [23] and Gruenberger et al., [54], in which they found that no statistically significant correlation was observed between grade of differentiation and IHC expression of HER-2 in CRC. On the other hand, a significant correlation between histological grade and IHC expression of HER-2 expression in CRC was reported in the studies done by Ghaffarzadegan et al., [56], and Deng et al., [57] reported an association between Her2/neu over expression and high grade.

As regard the correlation between IHC expression of HER-2 in tumor cells and lymph node metastasis, 93.3% of cases with positive lymph node metastasis were negative IHC expression of HER-2. No statistically significant link was reported (\(p > 0.05\)), and this agreed with results obtained by Mckay et al., [44] who found that HER-2/neu was expressed in 81.8% of tumors. But they did not find any correlation between HER-2/neu staining and lymph node metastasis. On the contrary, Park et al., [58] found overexpression of HER-2/neu in 12.5% of the studied cases. Tumors which showed positive HER-2/neu showed higher rate of nodal metastasis and Demirbas et al., [59] reported an association between Her2/neu over expression and lymph node metastasis. This difference can be explained by the relative small sample size.

As regard the correlation between IHC expression of HER-2 in tumor cells and TNM stage, 88.2% of cases with advanced stage (III & IV) were negative IHC expression of HER-2. No statistically significant correlation was reported (\(p > 0.05\)). This agreed with results obtained by Abd El All et al., [60] have demonstrated overexpression of HER-2/neu in 46.8% of CRC cases, but without significant association between TNM stage and HER-2/neu ex-presision. In contrast, Spano et al., [61], reported over-expression of HER-2/neu in CRC patients and its significant association with TNM staging. This difference can be explained by the relative small sample size.

As regard the relation between IHC expression of HER-2 in tumor cells and lymphovascular invasion, 85.7% of cases with positive lymphovascular invasion were negative for IHC expression of HER-2. No statistically significant link was reported (\(p > 0.05\)). This agrees with Elwy et al., [62] and Karaca et al., [63] who studied the expression of HER-2/neu on 192 CRC cases, and reported that there is no statistical significance between HER-2/neu and lymphovascular invasion. In contrast, Demirbas et al., [9] reported an association between Her2/neu over expression and lymphovascular invasion. There were several positive reasons for discrepancies between results, the most likely reason for the divergent finding of the antibodies is the different scoring system, the sensitivity of the antibodies used makes the comparison studies very challenging.

As regard the correlation between IHC expression of HER-2 in tumor cells and tumor budding, 77.7% of cases with high grade budding were negative IHC expression of HER-2. No statistically significant correlation was detected between HER-2/neu expression in tumor cells and tumor budding (\(p > 0.05\)). This consistent with Qingguo et al., [43] who reported that, there was no significant correlation was observed between HER-2/neu expression and tumor budding. In contrast to Shiento et al., [17] and Hase et al., [64], they showed statistical significance between HER-2 and tumor budding. It is important to note that all of these studies used different scoring systems and/or different antibodies than what is currently recommended, which makes comparative conclusions difficult if not impossible.
As regard the correlation between IHC expression of HER-2 in tumor cells and clinical outcome, 40% of cases with positive expression showed 5-year survival, 30% of cases were recurrent and 30% of cases were dead. Log-rank test showed no significant correlations between IHC expression of HER-2 and survival ($p>0.05$). Similar results are described in the study of Nathason et al., [45] who reported that, 33% of cases with positive expression showed 5-year survival and no significant correlations between IHC expression and survival by Log-rank test. In contrast to these results are four studies Osako et al., [50]; Kapitanvic et al., [66]; Lazaris et al., [67] and Saeki et al., [68] reported an association between Her-2/neu expression and survival. The most likely reason for this divergence is the technical variability in the performance of immunohistochemistry. It is well known that there are pitfalls in immunostaining for HER-2. Another reason may be due to the fact, that different antibodies have been used, stressing the importance of using standardized test systems most notably in case of therapeutic relevance of the results.

In the current study, among the examined 50 cases of colorectal carcinoma, 88% showed over expression ($>2$) of HER-2/neu. This agrees with Fabíola et al., [75] they found that there were statistical significant correlation between expression of HER-2/neu by qRT-PCR in tumor cells and tumor grade, depth of invasion and lymph node metastasis, and no correlation with histological type, tumor budding and lymphovascular invasion on their work on 360 cases of colorectal carcinoma. In contrast to these results, Li Q et al., [72] reported that, there were no a statistical significant correlation between expression of HER-2/neu by qRT-PCR in tumor cells and tumor grade, depth of invasion, lymph node metastasis, tumor budding and lymphovascular invasion on their work on 175 cases of colorectal carcinoma.

As regard the correlation between IHC expression of HER-2/neu by qRT-PCR in tumor cells and clinical outcome, 77.3% of cases with over expression of HER-2/neu by RT-PCR ($>2$) with advanced stages (III and IV) and this relation was statistically significant ($p<0.05$). This agrees with Pauletti et al., [69] they found that there was statistical significant correlation between expression of HER-2/neu by qRT-PCR in tumor cells and TNM stage of breast cancer.

As regard the correlation between expression of HER-2/neu by qRT-PCR in tumor cells and clinical outcome, 70.4% of cases with over expression of HER-2/neu by RT-PCR ($>2$) were disease-free survival for 5 years. Log-rank test showed significant correlations ($p<0.05$). This agrees with Pauletti et al., [69] and Field et al., [73] they showed statistical significance between HER-2 by qRT-PCR and survival rate of breast cancer.

As regard the correlation between expression of HER-2/neu by qRT-PCR in tumor cells and clinical outcome, 88% showed over expression ($>2$) of HER-2/neu by qRT-PCR while 12% showed under expression ($<2$) of HER-2/neu by qRT-PCR. These figures were similar to what was reported by Pauletti et al., [69] in which 83.5% of cases were over expression ($>2$) and 16.5% were under expression ($<2$) on their work on 125 cases of breast cancer.

As regard the correlation between expression of HER2/neu by qRT-PCR in tumor cells and some clinicopathological parameters (histological type, tumor grade, depth of invasion, lymph node metastasis, tumor budding and lympho-vascular invasion). There were a significant statistical positive correlation between HER-2/neu expression by qRT-PCR in tumor cells and above mentioned clinicopathological parameters ($p<0.05$). The studies done by Merkelbach et al., [70] and Benohr et al., [71] they found that there were statistical significant correlation between expression of HER2/neu by qRT-PCR in tumor cells and tumor grade, depth of invasion and lymph node metastasis, and no correlation with histological type, tumor budding and lymphovascular invasion on their work on 360 cases of colorectal carcinoma. In contrast to these results, Roxburgh et al., [50] reported that, 33% of cases with positive expression showed 5-year survival, 30% of cases were recurrent and 30% of cases were dead. Log-rank test showed no significant correlations between IHC expression and survival. The most likely reason for this divergence is the technical variability in the performance of immunohistochemistry. It is well known that there are pitfalls in immunostaining for HER-2. No statistically significant correlation was detected between HER-2/neu expression by qRT-PCR and IHC expression of HER-2/neu in CRC ($p>0.05$). This agrees with Massimo et al., [75] they found that, 66% of cases with negative expression of HER2/neu by IHC were associated with over amplification of Her2/neu by qRT-PCR with no statistically significant correlation. In contrast to
Fabiola et al. [74] reported that HER2/neu transcript levels were significantly lower in cases with low protein expression (0 or 1+) than in cases with high expression (3+).

It was found that, 20% of cases presenting with score 0 or 1+ showed overexpression by qRT-PCR, 58.3% of cases comprising 2+ immunostaining showed overexpression by qRT-PCR and this phenomenon was similar to findings in breast cancer where gene amplification is highly correlated to protein overexpression [76]. The differences between the current study and other studies may be due to the relative small sample size. Q-RT-PCR is now considered the gold standard for mRNA quantitative evaluation and its application to HER2 status evaluation could contribute to method standardization and reduce reports of variability.

Q-RT-PCR is a true quantitative technique, it is assessable according to Clinical and Laboratory Standards Institute guidelines and it is highly sensitive and specific because primers and hybridization probes are sequence specific. Therefore, applied this technique to fresh frozen and FFPE breast cancer specimens [75].

References


الأهمية التضخم الجيني وظهور الدالهة في سرطان القولون

أدى سرطان القولون إلى زيادة في السرطانات الشيوعية في الرجال والنساء. في الولايات المتحدة الأمريكية تم تشخيص حوالي 116% حالة من بينهم 100% حالة شهدة سرطان القولون والباقى السرطان المستقيم كما يشتبه حالات الوفاة 518% حالة. وفي مصر طبقاً إحصائيات عينت الأدوار، فإن سرطان القولون يشكل 43% من إجمالي السرطانات، وصل نسبة إلى 16.8% من سرطانات الجهاز الهضمي. وبعد الرايز شيعاً بعد الأورام الليفيوبلاستيك وأورام الثدي والكبد. يشير نمو من مستقبلات التراثي كيناز عبر الفاشية التي فمن الناحيةINTRICATE، إن الملف العام هو تغيير معدل التضخم الجيني وظهور الدالهة في سرطان القولون

باستخدام صبغة المناطعة الهيستوكيماوية وسيلة البلمرة المشتركة.

تطرق البحث وأهدافه: أجرت هذه الدراسة لتقييم الاعتدام من سرطان القولون في 50 حالة من حالات سرطان القولون المستقيم سابقاً وتشخيصات

تم الحصول عليه من كل الباحث، حيث يتم منطقة من 10 إلى 100% تكبير 10% حتى نوفمبر 2012.

وقد تم تحضير الشرائح، وأعد شريحتين لكل من العينات المبردة في الغلايا المغمورة في المريض بكم 0.5 ميكرون لكل شريحة إضافياً لفحص الميكروكوبية باستخدام صبغة الهيستوكيماوية والأغلى كما تم تجربة الأخرى بالصيغ الشيكة الهيستوكيماوية.

لكشف من خبر تيتو. وقد تم استخلاص الجين تيتو من العينات وتقدير كمية من طريق تقاطع البلمرة المشتركة.

تم دراسة الحالات وتحليلها إحصائياً بالنسبة للعمر، الجنس، مكان الولادة، جودة الحمض، وسمنة البالغة المثلثة ودراسة أربطة

بيضات الدائرة الهيستوكيماوية وسيلة البلمرة المشتركة. التفسير الإحصائياً لكل نتائج ما أن كان دلالة لا قد تم توثيقه.

نتائج البحث: تجاوز أعظم المرضى من 50 إلى 70 عام بمتوسط عمر 63.91 سنة ووتوات 12% من الحالات 4.8 عاماً. وأظهر

التوتر في المرضى في هذه الدراسة أن عدد الحالات 24 حالة بنسبة 48% أما حالات الإناث 27 حالة بنسبة 52%.

أظهرت الدراسة أن أتباع أنواع سرطان الدم هو سرطان الدم بنسبة 84% ثم سرطان الدم المخاطي بنسبة 87%، ومن حيث درجة

التبالاً في حالة 42 حالة 20% متواضعة التباين، و37 حالة 16% سادة التباين، ومن حيث مراحل تطور الورم تتعا عبا المرحلتي 5 إم، وجدت بالمرحلة (أ) 4 حالات (22%)، (ب) 24 حالة (40%)، (ب) 18 حالة (16%)، (ب) 18 حالة (16%)، (ب) 18 حالة (16%)، (ب) 18 حالة (16%)، (ب) 18 حالة (16%). وقد أخذ في الاعتبار الصيغة الفاشية فقط.

لم تكن هناك إحصائيات ذات إحساسية بين التعبير المناعي الهيستوكيماوي لدالهة تيتو في الخلايا السرطانية والنوع النسيجي، مكان الولادة، درجة التباين، درجة تتبع الورم، مدة التلاقي، التربة، البرامج البيولوجية، البرامج البيولوجية، البرامج البيولوجية، البرامج البيولوجية، البرامج البيولوجية، البرامج البيولوجية، البرامج البيولوجية، البرامج البيولوجية.

وقد وجد أن هناك علاقة إحصائية بين تعبير تقاطع البلمرة المشتركة لدالهة تيتو/نسو في الخلايا السرطانية والنوع النسيجي، درجة التباين، درجة انتشار الورم، مدة التروية الكيميائية، مدة التروية الكيميائية، مدة التروية الكيميائية، مدة التروية الكيميائية، مدة التروية الكيميائية.

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