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Basic Science

Association of Her-2 Expression and Clinicopathological Parameters in Colorectal Carcinoma in Indian Population

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Abstract

BACKGROUND: Human epidermal growth factor receptor 2 (HER-2) is an oncogenic gene and a well-established therapeutic target in several cancers including breast and ovary.

AIM: The present study aimed to compare HER-2 expression status with histological grades as well as Clinicopathological parameters including age, bleeding per rectum, pain/burning sensation in defecation and exercise.

METHODS: Her-2 status was assessed by immunohistochemistry (IHC).

RESULTS: Results of the study shows that 40.96% patients were Her-2 positive for expression and a statistically significant difference (p-value = 0.004) was observed in histological grades where most of the cases were of grade II. We also observed a significant difference in histological grades with gender (p-value = 0.04), as well as in both the age groups ≤ 55 years and > 55 years (p-value = < 0.0001). Patients with the bleeding rectum and pain/burning sensation in defecation had grade II/III tumours (93.4%, 88.7%) respectively. A significant association was observed between bleeding per rectum and pain/burning sensation in defecation. About 95% of patients with pain/burning sensation in defecation had bleeding per rectum.

CONCLUSION: To conclude, Her-2 can be a potential prognostic marker in CRC. The role of age, tumour grade and bleeding per rectum/burning sensation in defecation are of significant worth. Thus, CRC cases of high grades can be screened for HER-2/neu positivity so that they can be subjected to mAb-based individualised therapy.

Introduction

Colorectal cancer is the third most widespread malignancy globally affecting both the genders, with an estimated 1.4 million new cases annually. It accounts for about 9.7% of all cancer [1]. In the earlier studies, it was observed that HER-2 is associated with tumorigenesis, metastases of disease and poor clinical prognosis. The ligand binding causes receptor dimerisation and passes signal by auto-phosphorylation of HER-2 tyrosine kinase domain and activates the target proteins, such as mTOR, Src, STAT, MAPK [2]. In tumour cells, the Her-2/neu gene is amplified in approximately 20% to 25% of all cases instead of having twin copies per cell; there may be as many as 50 or 100 c-erb-B2 gene copies per cell [3]. This gene amplification even results in overexpression of Her-2/neu at both the mRNA and protein levels; amounting approximately 2,000,000 Her-2/neu molecules in a single tumour cell, instead of the 20,000 to 50,000 molecules per cell found in normal cells. When Her-2/neu is overexpressed at these abnormally high levels, the kinase activity becomes constitutively activated possibly due to auto-activation of accumulating Her-2/neu molecules [4].

The overexpression of Her-2/neu protein on the cell membrane leads to hypersensitivity of the cancer cell to the growth factors. Thus, it acts as a proto-oncogene. The role of EGFR (HER-2) is well established in breast cancer pathogenesis and is also one of the most important prognostic marker and target for the gene related therapy in breast cancer management. HER-2 overexpression in malignant cells intensifies metastatic potency and invasiveness [5]. The role of HER-2 in carcinogenesis has been

established in a rodent model by both *In vitro* as well as *In vivo* ways by the formation of tumours upon HER-2 amplification. Some recent studies highlighted novel mechanisms of HER-2 regulation that can be targeted for the treatment of cancer [6], [7].

Thus, the present study aimed to evaluate the immunohistochemical expression of HER-2/neu and its correlation with histological grade as well as clinicopathological parameters in colorectal cancer. The outcome of the study will help in establishing Her-2/neu as a potent prognostic marker for CRC and further helps in the management of Her-2 positive cases of CRC with any single treatment regime solely based on Her-2 expression, which again directs us towards the need of individualised therapy. Secondly, the association of Her-2 expression with clinicopathological parameters will help in establishing a better diagnosis and understanding aetiology of the disease.

Material and Methods

The present study was conducted at the Medical Biotechnology Laboratory, Department of Biotechnology, Jamia Millia Islamia, New Delhi, India after ethical approval from the Institutional Ethics Committee (17/9/10/JMI/IEC/2015). In the present study 83 (Eighty-three) unrelated subjects of Indian origin were included which were histologically diagnosed with colorectal cancer.

A tissue biopsy/colectomy sample was collected from each patient after taking their consent, and their tissues were stored in 10% Buffered Formaldehyde (Merck) at room temperature for immunohistochemical analysis. The adjacent non-cancerous tissue of the biopsies was taken as control for the IHC analysis. For the study purpose, the relevant clinical history of each case was collected in the pretext perform for correlation with clinicopathological parameters.

The tissue biopsy was processed for making paraffin embedded blocks. Further, the thin sections of size (4 µm) from all cases were cut by using ultrathin microtomy. All the cases were routinely processed, and Haematoxylin & Eosin staining was done for diagnosis and histological grading. All these cases were graded into one of three histological grades using WHO criteria as Grade I/II/III (well/moderate/poorly differentiated) respectively. Following the routine histological examination, these cases were further studied for expression of proto-oncogene Her-2/neu using Immuno-histochemical stains.

The Immuno-histochemical stains were performed using Avidin-Biotin technique [8]. Thin sections of paraffin block were cut using microtome and taken on poly-L-lysine coated slides. The sections were deparaffinized in three changes of xylene for 5 minutes each. Three changes of acetone were given for 5 minutes each. The slides were then washed in running tap water for 2-5 minutes. The slides were then given three changes of P.B.S. buffer. 0.03% H_2O_2 was prepared in methanol, and then the slides were kept in this solution for 30 minutes on a shaker. The peroxide block was discarded, and then three changes of P.B.S. buffer was given. Antigen retrieval was done by the microwave oven method [9]. After antigen retrieval, blocking was done using BSA (Merck). Immunohistochemistry is the technique utilised for the study of localisation of antigens in tissue sections using labelled antibodies as specific reagents through antigen-antibody interactions. The primary antibody for Her-2/neu was, Purified mouse anti-human Monoclonal antibody from (Novacastra, USA); the secondary antibody, tertiary antibody and DAB used was from (Novacastra, USA)

Stained slides were evaluated independently. Whole tumour area was observed, and overall percentage positivity of tumour cells for Her-2/neu has counted under x 400 magnification. For Her-2/neu, membranous staining was taken as real positivity. Based on the percentage of positive cells, Her-2/neu cases were graded into one of three grades as follows: Grade I: < 30% tumour cells positive, Grade II: ≥ 30% tumour cells positive (showing membranous as well as cytoplasmic staining), Grade III: ≥ 30% tumour cells positive (showing only membranous staining).

The Image J program and it's IHC profiler plugin (based on colour deconvolution) was used for analysis of captured images. The results are in the form of a histogram depicting count and intensity peaks as well as a log file characterising the negativity/positivity, its magnitude and % categorical values accordingly.

Considering a large number of similar values in the categorical data analysis was done. The expression of HER-2 was divided into positive and negative cases. The differences between the groups were studied by Fischer's exact test and other appropriate algorithms. All the statistical analysis was done by using SPSS 16 software.

Results

In the present study, a total number of 83 cases of colorectal carcinoma were studied. The age range (43) of all the cases in the study was between 30 to 73 years. The mean age of cases in the study group was 55.9 years, while the median age was 56 years and mode age came out to be 54 years. So, we

divided patients into two groups ≤ 55 years and > 55 years of age (Table 1).

Table 1: Clinicopathological characteristics of patients

Gender	Age	Bleeding per Rectum	Pain/Burning Sensation in Defecation	Exercise
Variable: n (%) {Total samples-83}				
Male: 63 (75.9 %)	≤ 55 years: 38 (45.8 %)	Yes: 76 (91.6 %)	Yes: 80 (96.4%)	High: 10 (12.0 %)
Female: 20 (24.1 %)	> 55 years: 45 (54.2 %)	No: 7 (8.4 %)	No: 3 (3.6 %)	Low/Moderate: 73 (88.0 %)

A significant difference was observed in the distribution of histopathological tumour grades among genders (p = 0.04). In both genders, grade II a tumour was most common. CRC patients in ≤ 55 years of age group, were mostly in grade I (31.6%) and grade II (63.1%), only 5.3% patients were in grade III.

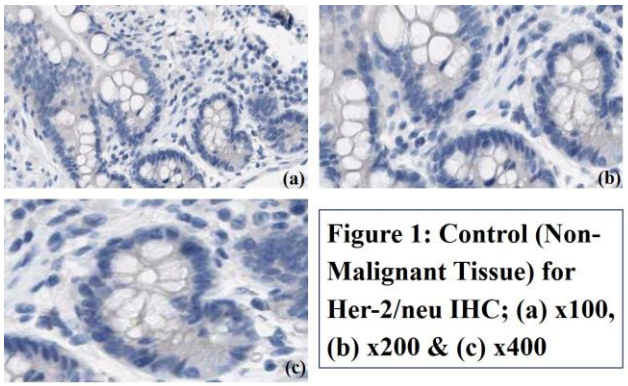

Figure 1: Control (Non-Malignant Tissue) for Her-2/neu IHC; (a) x100, (b) x200 & (c) x400

Patients in > 55 years of age group showed that patients were in grade II (64.4%) and III (35.5%) and the difference among age groups were found to be significant (p < 0.0001) (Table 2).

Table 2: Association of gender and age with histological grades in CRC patients

N = 83	Grade I	Grade I	Grade III	p-value
Male (63)	7 (11.1%)	39 (61.9%)	17 (27%)	0.04*
Female (20)	5 (25%)	14 (70%)	1 (5%)	
≤ 55 years (38)	12 (31.6%)	24 (63.1%)	2 (5.3%)	< 0.0001*
> 55 years (45)	0 (0%)	29 (64.4%)	16 (35.5%)	

It has been observed that cases which reported bleeding with rectum were either grade II/III tumours. However, patients who do not report bleeding were of grade I a tumour, and the difference among them was found to be significant (p < 0.0001).

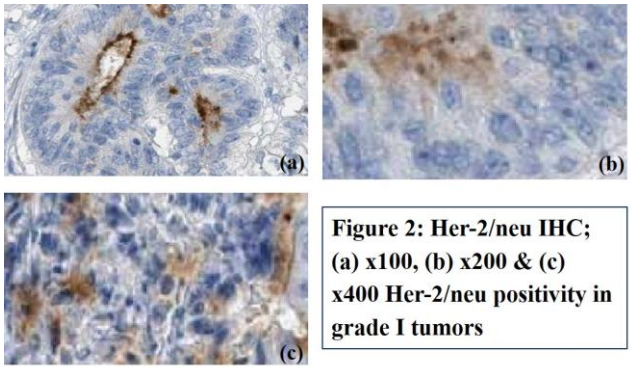

Figure 2: Her-2/neu IHC; (a) x100, (b) x200 & (c) x400 Her-2/neu positivity in grade I tumors

Patients who had burning sensation in defecation were either grade II/III (88.7%) however, patients who do not report burning sensation in defecation were of grade I a tumour (100%), and the difference among them was found to be significant (p = 0.002) (Table 3).

Table 3: Association of bleeding per rectum and pain/burning sensation in defecation with histopathological grades in CRC patients

Clinical status	Grade I	Grade II + Grade III	p-value
Bleeding per rectum			
Yes (76)	5 (6.6%)	71 (93.4%)	< 0.0001*
No (7)	7 (100%)	0 (0%)	
Pain/Burning Sensation in Defecation			
Yes (80)	9 (11.3%)	71 (88.7%)	0.002*
No (3)	3 (100%)	0 (0%)	

A significant association (p = 0.0004) was observed between burning sensation in defecation with bleeding per rectum.

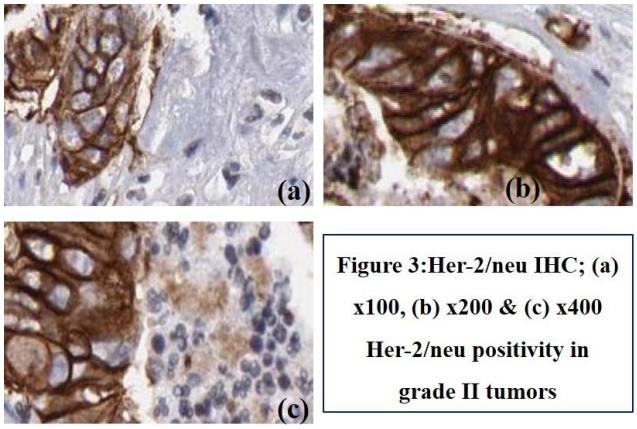

Figure 3:Her-2/neu IHC; (a) x100, (b) x200 & (c) x400 Her-2/neu positivity in grade II tumors

In the study group, it has been observed that most of the patients had bleeding per rectum (95%) with pain/burning sensation in defecation, only 5 % did not show any bleeding per rectum, however bleeding per rectum was also not observed in patients who did not show any pain/burning sensation in defecation (Table 4).

Table 4: Association of pain/burning sensation in defecation with bleeding per rectum in CRC patients

Clinical status	Pain/Burning Sensation in Defecation		p-value
Bleeding per rectum	Yes (n = 80)	No (n = 3)	
Yes (76)	76 (95%)	0 (0%)	0.0004*
No (7)	4 (5%)	3 (100%)	

CRC patients with a high level of exercise/physical activity showed no significant difference in the distribution of tumour grade in comparison to patients with low/moderate levels of exercise/physical activity but, it can also be seen that the number of patients with high level of activity (10/83, 12%) is very less in comparison to the patients with low/moderate level of physical activity (73/83, 88%). Though physical activity level didn't show any significant difference in the distribution of tumour grade, in the present study group, it was observed

that physical activity is helpful in preventing the onset of cancer as cited in previous research studies (Table 5).

Table 5: Association of exercise with histopathological grades in CRC patients

Exercise	Grade I	Grade II	Grade III	p-value
High (10)	1 (10%)	8 (80%)	1 (10%)	0.51
Low/Moderate (73)	11 (15.0%)	45 (61.6%)	17 (23.4%)	

Histological sections of 83 colorectal tumour cases were incubated with anti-Her-2/neu purified mouse anti-human monoclonal antibody. It was found that 49 (59.04%) cases failed to demonstrate any staining while the remaining 34 (40.96%) cases stained positive for Her-2/neu.

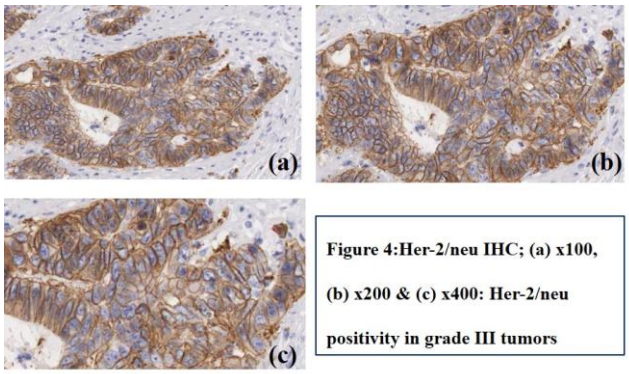

Figure 4:Her-2/neu IHC; (a) x100, (b) x200 & (c) x400: Her-2/neu positivity in grade III tumors

It was found that Her- 2/neu expression was positive in 2 (16.7%), 19 (35.9%) and 13 (72.2%) cases among grade I, grade II, and grade III tumours respectively and the differences were found to be significant (p = 0.004) (Table 6).

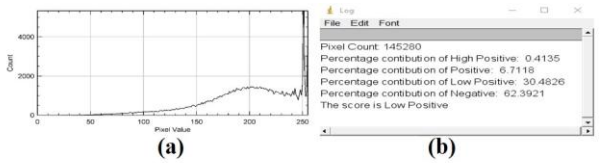

Figure 5.1: Grade I (IHC+) analysis from Image J program; (a) Histogram, (b) Log File

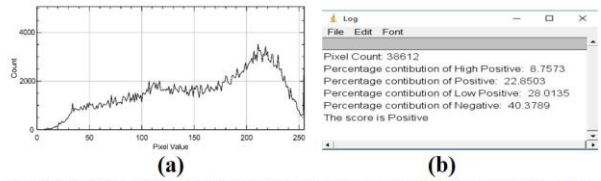

Figure 5.2: Grade II (IHC+) analysis from Image J program; (a) Histogram, (b) Log File

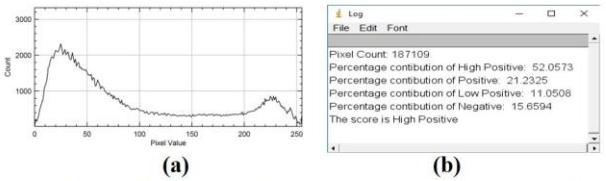

Figure 5.3: Grade III (IHC+) analysis from Image J program; (a) Histogram, (b) Log File

The Image J program and it's IHC profiler plugin (based on colour deconvolution) was used for analysis of captured images. The results are in the form of a histogram depicting count and intensity peaks as well as a log file characterising the negativity/positivity, its magnitude and % categorical values accordingly. Immunohistochemical expression observed was validated with Image J program and the results were found to be in concordance.

Table 6: Distribution of Her-2 immunohistochemical expression status among different tumour grades of CRC patients

Variables	Her-2 +ve (n = 34)	Her-2 -ve (n = 49)	P value
Histopathological grade			
Grade I (12)	2 (5.9%)	10 (20.4%)	
Grade II (53)	19 (55.9%)	34 (69.4%)	0.004*
Grade III (18)	13 (38.2%)	5 (10.2%)	

Discussion

Colorectal cancer is the third most common malignancy globally affecting both the sexes, with an estimated 1.4 million new cases annually. With global urbanisation and economic transition, adoption of western dietary habits and lifestyles and increasing life expectancy in developing nations, the incidence of colorectal cancer is rising steeply. An increasing trend in the incidence rates of Colorectal cancer in India has been reported from the various population-based studies.

Colorectal cancer is a multifactorial disease with various etiological and medical physiognomies. Age, personal history, dietary habits and lifestyle are the major risk factors for colorectal cancer. The chances of CRC diagnosis raises after 40 years of age, gradually increasing from age 40, rise steeply after 50 years of age [10].

In the present study, all 83 cases of colorectal carcinoma were distributed in the range of 30 to 73 years. In our study group, there were 38 cases in ≤ 55 years of age, and 45 were in > 55 years of age. Hence the prevalence rate showed a positive trend with an increase in the age of patients.

The mean, median, and mode age in our study come out to be 55.9, 56 and 54 years respectively. According to National cancer registry programme, hospital-based cancer registry, 2012 the mean age at diagnosis is 45 years in Indian population which is similar to mean age observed in developed nations of the west as stated by National Cancer Institute, USA. Over-expression of Her-2/neu has been notably associated with increased cellular survival, increased proliferation and decreased the apoptotic potential of cells leading to malignant transformation and maintenance of the associated malignancy [11]. A very scarce data is available particularly from Asian and south-east Asian region to indicate expression of Her-2/neu in patients with colorectal adenocarcinomas.

Overexpression of Her-2/neu in colorectal cancer shows a wide range of variability between 0-84% in different studies [12]. In neoplastic cells, dysregulation of the mentioned pathways and also increased expression of HER2/neu promotes tumour cell growth, progression and migration [13]. Overexpression of the HER2/neu receptor is detected in 25%-35% of breast cancer patients and has been known as an important prognostic and predictive factor [14]. In the present study, the overall expression rate of Her-2/neu was 40.96% which was confined mainly in the membrane with intermittent cytoplasmic localisation. This was in accordance to few studies which reported both membranous as well as cytoplasmic overexpression with rates streaking up to the tune of 60% [19]. On the contrary, most of the studies reported the rate of membranous overexpression between zero and 15% [17], [19]. According to histological grade, its expression varied from 16.66% (2/12) in grade I tumors to 72.22 % (13/18) in grade III tumors. Thus, Her-2/neu expression was positively related to histological grade, and the difference in expression rate across different grades was statistically significant p-value = 0.0045 (*, $p < 0.05$). Our results are in concordance with previous studies of [17]; these studies also showed very similar positive correlation between Her-2/neu expression and histological grades in their respective studies.

This positive correlation between Her-2/neu expression and histological grade accounts for the poor prognostic significance of Her-2/neu in case of colorectal carcinoma. Based on these findings only this gene has become a target for the successful management of CRC. Further, in the present study group, most of the cases reported bleeding per rectum and pain/burning sensation in defecation showed to have grade II/III a tumour and those who do not report was found to be in grade I a tumour. Hence, pain/burning sensation in defecation and bleeding per rectum are important indicators for high-grade CRC.

It has been reported that the (grade II) moderately differentiated carcinoma was the most common type and explained that the variation between different studies be related to the randomised selection and also small sample size [18], [20]. In clinicopathological parameters role of age, tumour grade and bleeding per rectum/burning sensation in defecation are significant in the diagnosis of CRC. Further, the Her-2 positive cases especially with high grades of CRC can be subjected to mAb-based individualised therapy targeting Her-2/neu. Therefore, we hypothesise that Her-2 may be a potent prognostic marker in CRC and can play a pivotal role in the better management of the disease.

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