THE ROLE OF HER-2 CYTOPLASMIC RECEPTOR OVEREXPRESSION IN COLORECTAL CANCER

***Beraru Ilie, Szegedi Ladislau**

*University of Oradea, Faculty of Medicine and Pharmacy, 10 December 1st, Oradea
iliuta_beraru@yahoo.com

Abstract

There is still no consensus on the incidence, role and overexpression of Her-2 receptor cytoplasmic localization in colorectal cancer. Objectives: Our study aims to identify the incidence and effect of this overexpression on the development of colorectal cancer patients hospitalized in the Surgical Clinic of the Emergency County Hospital Oradea. Material and Methods: We studied patients undergoing extirpation of the colon or rectum in 2008-2010 for colorectal cancer at various stages. In addition to demographics, severity and clinical evolution were studied immunohistochemically biopsy sections from tumors excised. Results: The incidence of HER-2 cytoplasmic overexpression (2+ and 3+) in the study group was 46.9%.The incidence of recurrence showed a significant difference between the Her-2 positive and negative (41.9% vs 58.1%, p = 0.00)7. Conclusions: The incidence of Her-2 overexpression cytoplasm is high in our region, but remains within the limits observed in literature.

Key words: colorectal, cancer, immunohistochemistry, Her-2, cytoplasmic.

INTRODUCTION

Colorectal cancer is a major health problem worldwide, with an annual incidence of 1.2 million and an annual mortality of over 600,000 people (Ferlay J.et al., 2008; Winawer S.J., 2007). One of the reasons for the relatively high mortality is advanced tumor stage at diagnosis, the presence or symptoms caused by the lack of non-specific symptoms during the initial stages of the disease. With the advancement in the evolution of tumor recurrence after resection risk increases, the chances of survival decrease. Chemotherapy has proven to be an effective strategy as adjuvant therapy, but is not yet able to prevent recurrence in all patients (Andre T.et al., 2004). Therefore, many researches are under way to utilize adjuvant compounds as alternatives. Monoclonal antibodies and other biological products, targeting protein association tumor and tumor blocking essential processes are studied in detail. An important step in this process is the identification of tumor specific proteins that can be targeted by these compounds.

One of these compounds is *Her-2 receptor*, which is primarily associated with breast cancer (Wolff A.C., 2007). Several studies evaluating the *Her-2 receptor* in *colorectal cancer* led to a great debate last century because *overexpression* rates varied between zero and 84% (Ross J.S., 2001). The clinical significance of *HER-2 receptors* in these publications

was not consistent; some publications have associated *overexpression* of *Her-2 receptor* with survival (Kay E.W.et al., 1994; Lazaris A.C. et al., 1995; Osako T.et al., 1998), while others failed to find such a correlation (Berney C.R. et al., 1999; Kluftinger A.M.et al., 1992; Sun X.F. et al., 1995). Most researchers agreed that expression differences were probably due to differences in technical approaches, antibodies and scoring protocols, but conclusive data have not been presented before. Since then a large number of *immunohistochemical* studies have appeared on the *HER-2* oncogene in *colorectal cancer*. Most membrane showed *overexpression* rates between zero and 15% (Half E.et al., 2004; Li Q.et al.,2011;Schuell B.et al.,2006). Remarkably, some studies have reported *overexpression* of both the cytoplasmic membrane and the much greater rates of up to 60% (Park D.I. et al., 2007; Kruszewska W.J. et al., 2010; Uner A.et al., 2005). So far, there is no consistency of opinion proportion *Her-2 receptor overexpression* in colorectal tumors.

If we separate the results of studies based on the location of *HER-2* oncogene, there is a clear pattern with approximately 5% of patients who have an *overexpression* membrane, and about 30% of all patients whith cytoplasmic *overexpression*. If cytoplasmic *Her-2* has a pathophysiological role in colorectal cancer, compounds targeting *Her-2* intracellularly, like lapatinib may be a therapeutic option for 360.000 patients per year, an important discovery for the treatment of colorectal neoplasms (Blok E.J.et al., 2013).

Given the lack of conclusive data about intracellular *overexpression* of *HER-2* oncogene and especially about its correlation with the clinical data we wanted to bring these questions by studying patients with colorectal cancer in our geographical area.

MATERIAL AND METHODS

The study was conducted in the Department of Surgery II Emergency County Hospital Oradea in January 2008 - December 2010. Upon approval of the Ethics Commission of the unit we selected patients who underwent resection of colorectal adenocarcinoma. None of the patients has undergone preoperative chemo- or radiotherapy. We excluded patients with familial adenomatous polyposis, multiple colorectal tumors, synchronous malignancy other than colorectal cancer.

From the harvested biopsy material during surgery,sections were obtained for *immunohistochemical* examination. The sections were mounted on slides, coated with a suitable adhesive. Slides were deparaffinized in xylene, rehydrated through alcohols gradually. Endogenous peroxidase activity was neutralized with aqueous hydrogen peroxide and methanol for

30 minutes at room temperature (25°C). The slides were rinsed with filtered water. The recovery of antigen was carried out by microwave treatment of the blade in citrated buffer (pH 6.0) for 4 minutes on high setting and then 6 minutes at low energy. Sections were washed in Tris buffered saline (TBS). Non-specific binding was blocked with streptavidin complex/ biotin - horseradish peroxidase for 15 minutes. Slides were incubated with monoclonal anti- *Her -2* antibody directed against the cytoplasmic domain of the *Her-2* receptor (CB clone 11 Ready -to -Use *Her-2*/c-erbB-2 oncoprotein Antibodies, Novocastra Laboratories Ltd., Newcastle, UK) at an optimal concentration for 2 hours. A second monoclonal antibody against *Her -2* was also used to stain tissues and cell lines control,to verify the expression and localization of *Her -2*. Immunoreactivity was detected using an immunoperoxidase method (Peroxidase Detection System Ready -to - Use, Novocastra Detection System User Manual, Novocastra Laboratories Ltd., Newcastle, UK). Chromogen 3,3' tetrahidroclorid diaminobenzidine (DAB Hi Novolink Polymer RE7270 - K, Novocastra Laboratories Ltd., Newcastle, UK) was then added and the color reaction was observed with the optical microscope (fig.1). The reaction was stopped by immersion in deionized water, and the slides were contrasted with hematoxylin and then mounted. As a control we used human breast cancer cells, were stained for *Her -2* membrane and the additional negative control was not added to the first antibody, but the remaining steps have been observed. Cases with less than 10% of tumor cells immunoreactive for *Her-2* and cytoplasmic staining with moderate (2+) and high (3+), are considered positive. Cases *Her-2* staining absence (0) or low intensities (1+) were found to be negative. *Her-2* categorization by location (membrane and cytoplasm) was made using the model of color:

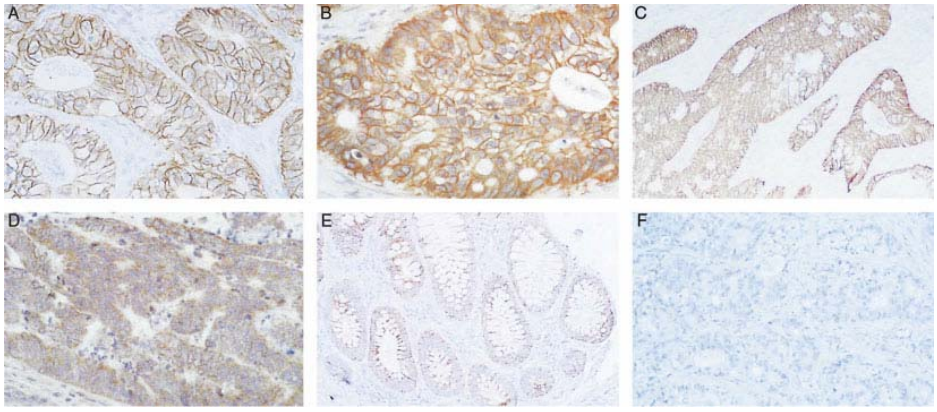

Fig. 1 The intracellular localization of Her-2
A - Membrane localization of Her-2; B, C - membrane and cytoplasmic localization; D - cytoplasmic localization; E - normal colonic mucosa cells with HER-2 cytoplasmic staining; M - Her-2 negative tumor cells

To confirm the histochemical method we checked 10 random samples of sections which showed intense staining (3+) for *HER-2* cytoplasmic staining method immunoelectron microscopy Gold visualized (fig. 2). The method consists in determining the tissue in phosphate buffered saline (PBS) with 2% paraformaldehyde and 0.2% glutaraldehyde to 4-6°C overnight. Dehydration was done in a series of ethyl alcohols, decreasing the temperature at -20°C. Thin sections mounted on nickel grids and the pre-treated with citrate buffer (pH = 6) at 100 ° C for 10 minutes and cooled 15 minutes. Non-specific binding was blocked with 0.1 M glycine for 15 minutes and PBS with 0.05% Tween 20 (PBST) for 15 minutes. Grids were incubated with the same monoclonal antibodies as in the case of *immunohistochemistry* for 3 hours at room temperature, and then washed in PBST and incubated for 60 minute particles of 10 nm colloidal gold (10 nm AuroProbe EM, Amersham Pharmacia Biotech Inc., N.J., USA). After rinsing in PBST and filtered water grids are enhanced with silver stain for 20 minutes. Sections were stained with uranyl acetate and lead citrate and examined with transmission electronmicroscopy. The location of the *Her-2* antigen was detected by the presence of colloidal gold particles:

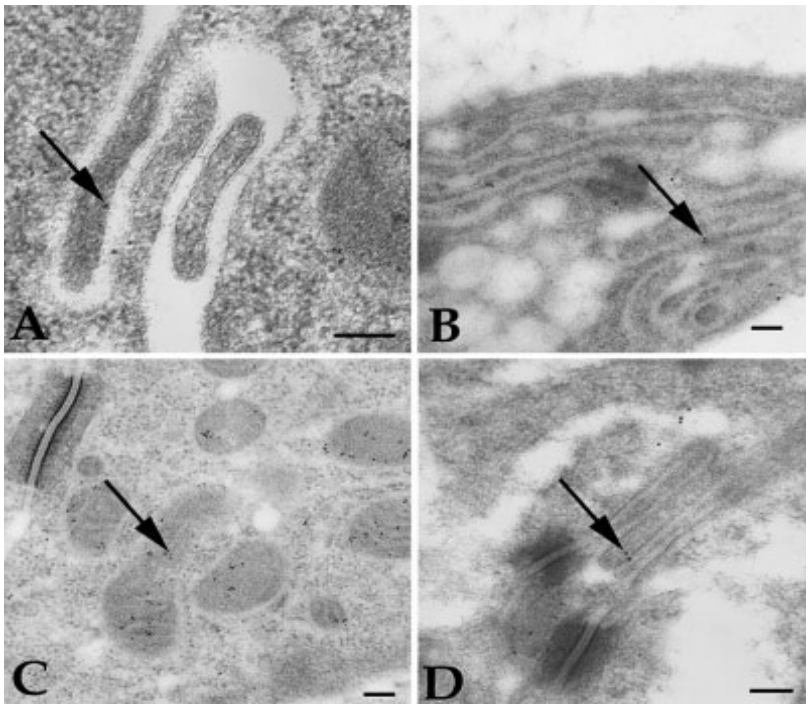

Fig. 2 Immunelectron microscopy-Gold method

The arrows indicate observed electronmicroscopy colloidal gold particles (line = 100 nm) coresponding to intracellular *HER-2* protein A,D - the plasma membrane in the intracellular; B - in the endoplasmic reticulum; C - in mitochondria;

The clinical criteria followed during hospitalization and follow-up period of 3 years were: sex, age, tumor location, tumor grading, TNM staging, the occurrence of relapse or death. Statistical analysis was performed using statistical software MedCalc ® version 12.5.0.0 Medical (MedCalc ® Software, Mariakerke, Belgium). Statistical test results will be represented by probability hypothesis "null" (p), its value below 0.05 shows a statistically significant difference between the groups studied.

RESULTS

Following the selection criteria resulted in a total of 209 patients, including 120 men and 89 women (p = 0.0380, chi-square test).

The number of patients with *Her-2* positive immunohistochemistry cytoplasmic was 98 (46.9%), of which 54 (25.8%) with high Ca2 + and 44 (21.1%) 3+ level. 3+ positive sections were confirmed by choosing 10 random samples were analyzed using electronmiscroscopy. All 10 sections showed positive staining for *Her-2* oncogene intracellularly. Clinical variables tracked by the presence or absence of *Her-2* cytoplasmic receptors can be summarized in the following table:

Table 1

Statistical Analysis

Clinical variables	Total number of patients	IHC pozitives (2+ şi 3+)	p (chi-squared test)
Age			
< 60 years	66 (31,6%)	27 (40,9%)	0,3040
> 60 years	143 (68,4%)	71 (49,7%)	
Sex			
M	120 (57,4%)	60 (50,0%)	0,3649
F	89 (42,6%)	38 (42,7%)	
Localization	41 (19,6%)	23 (56,1%)	
Ascendent	10 (4,8%)	3 (30,0%)	
Transverse downward	19 (9,1%)	6 (31,6%)	0,3426
Sigma	74 (35,4%)	34 (45,9%)	
Rectum	65 (31,1%)	32 (49,2%)	
Tumor Grading			
Well differentiated	7 (3,3%)	3 (42,9%)	0,5293
Moderate	192 (91,9%)	92 (47,9%)	
Low	10 (4,8%)	3 (30,0%)	
Level of invasion			
T1/T2	23 (11,0%)	12 (52,2%)	0,7514
T3/T4	186 (89,0%)	86 (46,2%)	
Lymph node metastases			
Absent	35 (16,7%)	16 (45,7%)	0,9738
Present	174 (83,3%)	82 (47,1%)	
Distant metastases			
Absent	163 (78,0%)	74 (45,4%)	0,5183
Present	46 (22,0%)	24 (52,2%)	
TNM staging			
I	34 (16,3%)	15 (44,1%)	
II	94 (45,0%)	41 (43,6%)	0,7257
III	35 (16,7%)	18 (51,4%)	
IV	46 (22,0%)	24 (52,2%)	

In evolution during 3 years follow, we describe the occurrence of relapse and death during this time - DFS (disease free survival) and overall survival - OS (overall survival)

Table 2

Survival Analysis

Survival	Patients number (%)	IHC positive (%)	p (chi-squared test)
DFS	181 (86,6%)	76 (41,9%)	0,0007
OS	200 (95,7%)	92 (46,0%)	0,3821

A more detailed analysis of the evolution we can get by logrank test and constructing Kaplan-Meier survival curves. Comparing IHC positive and negative patient's relapse-free survival we obtain the following results: hazard ratio = 1.9897 (0.91 to 4.32), p = 0.0945 and the following graph:

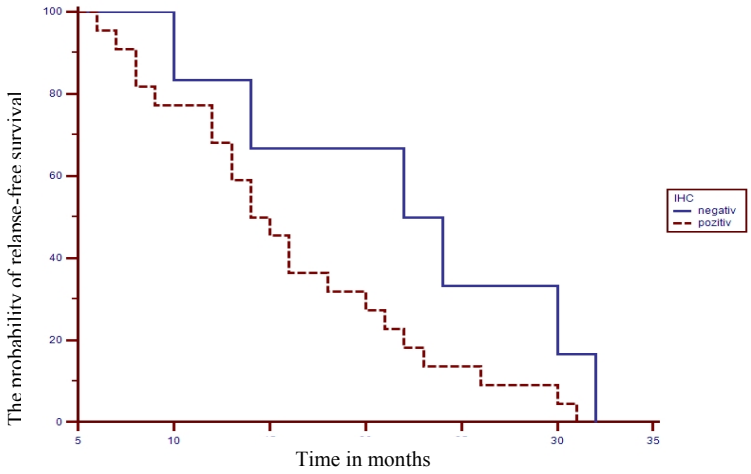

Fig. 3. The Kaplan-Meier survival curves

DISCUSSIONS

The incidence of *Her-2* cytoplasmic receptor *overexpression* in the study group was 46.9% which is within the values observed by the authors of studies.

From the results presented it is observed that cytoplasmic *overexpression* of *Her-2* was not significantly correlated with any clinical variable. Oncogene positive patients for this tumor are frequently presented with a particular location, in a later stage or have a poor differentiation. These results are consistent with several specific studies that also found no clinical correlations (Kountourakis P. et al., 2006; Kruszewska W.J. et al.,

2010), but other studies refute the observations that *overexpression* of this oncogene is associated with cytoplasmic tumoral differentiation (Half E. et al., 2004; Uner A. et al., 2005). The evolution of positive and negative patients demostrates (immunohistochimically) that there is a tendency of relapse in *Her-2* positive patients, but this tendency remains under statistical significance.

However Her-*2* cytoplasmic domain has a role in the prognosis of colo-rectal cancer,and it might also be involved in the pathogenesis of tumor and *Her-2* in breast cancer membrane. A plausible explanation is that the *Her-2* cytoplasmic form homodimers, which leads to an activation of the intracellular tyrosine kinase domain. Therefore, administration of trastuzumab would have no effect, because this concerns only the extracellular domain of the antibody. Lapatinib, an inhibitor of the intracellular tyrosine kinase, has recently been approved for treatment of patients with *Her-2*-positive breast cancer (Geyer C.E.et al.,2006). If *Her-2* cytoplasmic domain is indeed active in colorectal carcinogenesis, administration of lapatinib, or other component targeted for intracellular *Her-2*, may be an important therapeutic improvement, especially for this type of cancer.

CONCLUSIONS

Almost half of the patients in our study showed *overexpression* of *Her-2* cytoplasmic receptor, but this feature of the tumor was not significantly correlated with demographic clinical characteristics, severity or location of the cancer. Evolution encumbered by relapses was more common among *Her-2* -positive patients, but did not reach statistical significance. There was no oncogenic influence on mortality.

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