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Simultaneous estimation of c-erbB2 and p53 proteins – lack of clinical relevancy in colorectal cancer

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Introduction. Selected oncoproteins can be helpful for the prediction of more aggressive tumor behavior and can influence the choice of treatment modality and follow up. Simultaneous evaluation of more than one potentially prognostic factor allows for forming a more adequate prognosis.
Material and methods. c-erbB2 membranous overexpression was evaluated immunohistochemically together with the expression of p53 in 107 resected colorectal adenocarcinomas.
Results. 2+/3+ c-erbB2 overexpression was observed in 26 tumors (24%). p53 was positive in 62 cases (58%). In 16 cases c-erbB2 was overexpressed (2+/3+) together with positive p53. There were no statistically significant correlations between c-erbB2 and/or p53 expression neither in relation to each other nor to the commonly estimated pathoclinical features of the disease, nor to survival time.
Conclusions. Simultaneous estimation of c-erbB2 and p53 does not provide any prognostic information in patients with colorectal tumors.

Key words: c-erbB2, p53, colorectal cancer, prognosis

Introduction

Colorectal cancer is the second most common cause of cancer death in EU countries [1]. Molecular biology creates hopes for the recognition of all the mechanisms responsible for colorectal carcinogenesis and further progression of the disease. However, many interactions between molecular alterations and pathological and clinical features still remain unknown [2, 3]. Oncogenic proteins may accompany particular genetic alterations [2, 4-6]. The correlation between the expression of selected oncoproteins and the pathological and clinical features observed in neoplasmatic diseases have been the subject of many studies [2, 6, 7]. Observations of selected oncoproteins in different malignancies can be helpful for predicting more aggressive tumor potential or for determining its response to therapy. Blocking the receptor c-erbB2 protein of the *c-erbB2* gene (also known as *Her-2/neu*) on the surface of cancer cells with trastuzumab has become part of the standard management of breast cancer patients [8-10]. Interfering EGFR signaling with Cetuximab decreases drug resistance in the treatment of colorectal cancer, whereas blocking VEGF with bevacizumab inhibits neoangiogenesis in primary tumors and metastases of colorectal cancer [8, 11]. The presence of selected antigenic components of the members of epithelial growth factor receptor family on the cell surface of the primary and secondary tumors of the breast, colon and pancreas promotes studies on the immunotherapy of these neoplasms [6, 12-15]. Four different receptors with tyrosine kinase activity are included into the epidermal growth factor receptor family: c-erbB1 (EGFR), c-erbB2, c-erbB3 and c-erbB4, while c-erbB1 and c-erbB2 show significant structural similarity [13, 16]. c-erbB2 is a 185-kDA cell membrane glycoprotein. It plays a part of a regulatory switch between cell differentiation and proliferation of epithelial cells [13, 17]. c-erbB2 activity favors cells proliferation, prolongation of their survival time, and promotes neoangiogenesis and tumor dissemination [7, 9, 16]

c-erbB2 gene is located on chromosome 17, together with the *p53* suppressor gene, which is of great importance in oncogenesis. The expression of mutated p53 protein in primary colorectal cancer may be an indicator of poorer prognosis, although the prognostic value of p53 has not been clearly determined [20-22]. *p53* gene alterations can affect the chemo- and radiosensitivity of many neoplasms, including colorectal tumors [18, 23, 24]. Correlations between p53 and c-erbB2 expression and prognosis were observed in patients with breast cancer, and also in gastric cancer patients [25, 26]. It was found that *p53* gene mutation in breast cancer patients free of

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lymph node metastases has an independent negative prognostic value only in the presence of *c-erbB2* amplification [27].

About 1/3 of Dukes B colorectal cancer patients will experience disease recurrence [28]. In almost a half of the radically treated colorectal cancer patients distant metastases will occur in the follow up period [29]. c-erbB2 and mutated p53 proteins take part in colorectal oncogenesis and tumor progression [2, 30, 31].

The aim of this study was to estimate the prognostic value of simultaneously evaluated c-erbB2 and p53 proteins in surgically treated colorectal cancer.

Material and method

Patients

107 consecutive patients (45 women (42.1%) and 62 men (57.9%)) with T2–T4 resectable adenocarcinoma of the colon were enrolled in the study. The patients were operated in the Department of Surgical Oncology of the Medical University in Gdańsk between October 1997 and December 2000. Age range was 31-85 years, mean 64.8 yrs. median 67 yrs. The primary tumor was located in the colon in 53 cases, and in the rectum in 54 cases. The follow up period was completed by the end of November 2004. Eighty eight patients were treated by radical resection (38 with colon and 50 with rectal tumors). In this group we had performed 16 right and 5 left hemicolectomies, 17 sigmoidectomies, 34 anterior resections, 11 Miles' operations, and 5 Hartmann's procedures In 3/88 patients with solitary liver metastases radical metastasectomy was performed. Nineteen patients underwent palliative tumor resection: 12 presented with disseminated disease, and non-radical R1 resection was performed in 7 cases. Patients receiving neoadjuvant treatment were not enrolled into the study. Standard adjuvant therapy was administered depending upon the stage of the disease [32]. Median follow up was 48 months. Median survival time was 59 months. Of the 107 patients, 56 (52%) died (50 from colorectal cancer recurrence, 1 from dissemination of lung cancer as a second neoplasm, in 5 patients the cause of death remained unknown). The histological type of the tumor was evaluated according to the WHO classification [33]. Tubular adenocarcinoma was found in 91 cases (85%) and mucinous adenocarcinoma in 16 cases (15%). Histological differentiation was classified as G1 in 13 cases (12.1%), G2 in 64 cases (59.8%) and G3 in 30 cases (28.8%). All mucinous carcinomas were scored as grade G3 [34]. Staging was assessed according to pTNM classification [35]. There were 23 T2 tumors (21.5%), 77 T3 (72%), and 7 T4 tumors (6.5%). In 50 cases (46.7%) metastatic regional lymph nodes were observed (N1 – 31 (29%), N2 – 19 (17.7%)). Stage I disease was diagnosed in 17 patients (15.9%), stage II in 38 (35.5%), stage III in 37 (34.6%) and stage IV in 15 cases (14%).

Immunohistochemical analysis

Sections from formalin-fixed, paraffin-embedded material retrieved from the archives of the Department of Pathology of the Medical University in Gdańsk were evaluated for c-erbB2 and p53 proteins expression. Specimens without necrosis were chosen for imunohistochemistry. Staining was evaluated at the tumor base, at the point of the deepest tumor infiltration. For immunohistochemical staining of c-erbB2 we used the rabbit polyclonal antibody A 0486 (DAKO) diluted 1:40. Immunohistochemical staining for p53 was carried out with mouse monoclonal antibody M 7001 (clone DO-7) diluted 1:50. All the immunohistochemical procedures were performed according to the instructions recommended by the manufacturers. The presence of the antigen-antibody complexes was revealed by adding biotinylated secondary antibodies and the streptavidin-peroxidase complex (LSAB system, DAKO K0675). Diaminobenzidine (DAB) was used as chromogen. Additionally, the sections were counterstained with Meyer hematoxilin. According to the common technique the membranous staining was semiquantitavely scored estimating the c-erbB2 protein expression in tumor cells as 0, no staining or membrane staining in less than 10% of cells; 1+, incomplete membrane staining is observed in more than 10% of cells; 2+, a weak to moderate complete membranous staining detected in more than 10% of cells; 3+, a strong complete membranous staining detected in more than 10% of cells (14.36). Scores 2+ and 3+ were considered as protein overexpression. p53 expression was interpreted positive when nuclear staining of over 30 % cells was detected [37]. Figures 1 and 2 present consecutive cases with c-erbB2 and p53 overexpression.

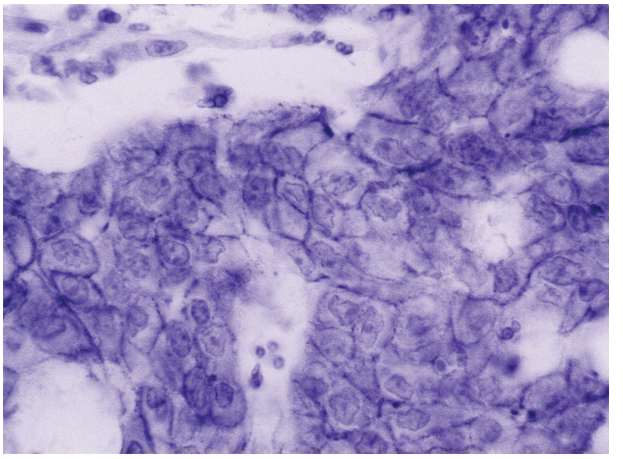

Figure 1. c-erbB2 membranous protein overexpression (3+) in cancer cells (x400)

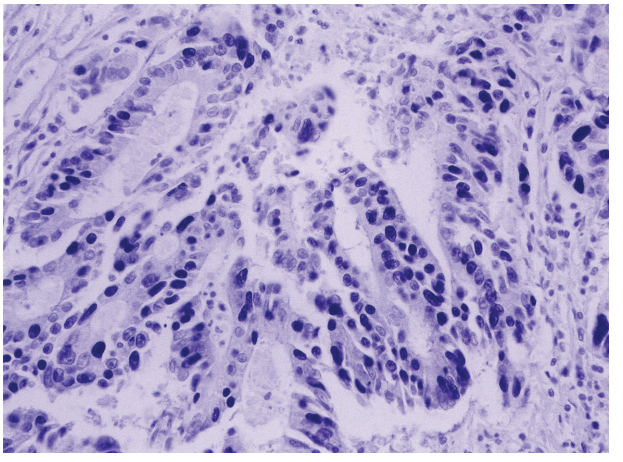

Figure 2. p53 protein expression in cancer cells (x200)

Statistical analysis

Data analysis was performed with the use of Statistica 7.0 (StatSoft Inc. Tulsa, OK, USA). The correlations between the investigated parameters (n=107) were tested by Pearson chi2 statistics with the Yates exact test correction ($p<0.01$ for statistically significant differences). Survival analysis (n=88) was performed with the Kaplan-Meier method and compared

using the log-rank test ($p<0.05$ for statistically significant differences).

Results

We observed no membranous c-erbB2 protein expression (grade 0) in 49 cases (45.8%), grade 1+ in 32 cases (29.9%), grade 2+ in 20 cases (18.7%), and grade 3+ in 6 cases (5.6%). Overexpression (grade 2+ and 3+) was present in 26 primary tumors (24.3%). p53 protein was expressed in 62 specimens (57.9%). Both proteins: c-erbB2 overexpression and p53 positivity were observed in 16 cases (15%). There were no statistically significant correlations between c-erbB2 and/or p53 expression, neither in relation to each other nor to the commonly estimated pathoclinical features of the disease, such as tumor location, WHO tumor type, depth of bowel wall infiltration, regional lymph node involvement, distant metastases, tumor differentiation and patient age and gender (n=107). We observed some slight tendencies between more frequent mutual c-erbB2 overexpression and p53 among younger patients (p=0.07), in case of mucinous adenocarcinoma (p=0.08), and in those cases when the tumor was located in the rectum (p=0.06) (Table I). The univariate analysis performed in the group of radically treated patients (n=88) did not show any correlation between the tested protein expression and survival time, also in the cases of mutual expression of p53 and c-erbB2 (Table II). This observation rendered the multivariate analysis using Cox's multiple regression model useless in the course of our study.

Table I. Selected pathoclinical features in relation to the mode of c-erbB2 and p53 expression (n=107)

	0	1	2	p value
age ≤ 67 years	17	25	9	
age >67 years	18	31	7	p=0.07
colon	23	24	6	
rectum	12	32	10	p=0.06
adenocarcinomas	29	51	11	
adenocarcinoma mucinosum	6	5	5	p=0.08

0 – c-erB2 0/1+ and p53 negative
1 – c-erbB2 2+/3+ and/or p53 positive
2 – c-erbB2 2+/3+ and p53 positive

Table II. Univariate analysis of investigated features (n=88)

Variable	p value
c-erbB2 0/1+ vs. c-erbB2 2+/3+	0.75
p53 (-) vs. p53 (+)	0.26
p53 (+) and c-erbB2 2+/3+ vs other combinations	0.78

Discussion

The routine prognostic factors derived from TNM classification do not allow for the precise prediction of the course of disease nor for the estimation of tumor cell sensitivity to adjuvant therapy in radically treated colorectal cancer patients [29]. It is likely, that the variety of alternative pathways in colorectal carcinogenesis and intratumor genetic heterogeneity are responsible for the different course of the disease in patients with pathoclinically similar colorectal tumors [3, 38]. The recognition of additional factors responsible for primary tumor development and disease dissemination can be helpful in planning proper treatment and follow up of colorectal cancer patients. The determination of the role of membranous c-erbB2 overexpression in ductal breast cancer as an indicator of more aggressive cancer behavior and of the sensitivity to trastuzumab and other drugs has become a great diagnostic and therapeutic success [8-10]. The wide spread of unified methods for the evaluation of c-erbB2 protein expression, such as the HercepTest® has rendered research easier [14, 36, 39]. Clinical trials with trastuzumab have also been undertaken in colorectal cancer patients with c-erbB2 overexpression, and some clinical benefits have been observed [32].

The use of polyclonal antibodies A 0485 by DAKO provides similar results of membranous c-erbB2 evaluation as those observed after processing antibodies from the original HercepTest®, and the procedure is less expensive [40]. We applied the A 0485 DAKO antibodies in our study.

Due to the fact that membranous c-erbB2 over-expression in breast cancer cells is the result of *c-erbB2* gene amplification, FISH (*fluorescence in situ hybridization*) is commonly used to reveal the number of *c-erbB2* gene copies, and to confirm protein overexpression diagnosed immunohistochemically. Both FISH and immunohistochemistry are equivalent in case of 3+ c-erbB2 overexpression in breast cancer [39]. In 2+ cases there exists significant discrepancy of the results obtained with the use of the two methods, and complementary estimation by FISH to immunohistochemical evaluation of protein expression is recommended. However, this significantly increases the costs of diagnosis [39, 41]. Gene amplification may be also confirmed by PCR (*polymerase chain reaction*) [41]. Distinctly from breast cancer cells in the case of colorectal cancer cells with membranous overexpression of c-erbB2 the c-erbB2 gene amplification is rarely observed, or not observed at all. If gene amplification is observed in colorectal cancer cells, it does not necessarily correlate with c-erbB2 membranous protein overexpression [41-43]. Thereby the expensive FISH procedure appears not to be as valuable in case of this malignancy, as it is in breast cancer, and we have not performed it in the course of our evaluation. The mechanisms leading to c-erbB2 protein accumulation in breast and colorectal cancers differ, with suggestions of presence of alterations at the transcription level in case of the breast, and at the level of translation and

posttranslational events in the case of the colorectum [43]. However, there are also well documented reports stating that gene amplification precedes membranous c-erbB2 overexpression in colorectal cancer cells [41, 44, 45]. The authors of these reports have estimated protein overexpression at a level not exceeding 3% to 5% of cases.

According to many studies, membranous c-erbB2 overexpression in colorectal cancer cells is more rare than in breast cancer cells [14, 41-43]. Usually the authors determine between a few percent and anything between 10-20% of cases with membranous c-erbB2 overexpression [14, 32, 41, 42, 44-47]. However, there are also reports, where membranous c-erbB2 overexpression is determined in more than 40-60% of cases [6, 48]. These discrepancies may be, to some extent, explained by the diversity of antibodies and examination methods which are applied. The rarity of protein overexpression limits clinical trials on the efficacy of trastuzumab in colorectal cancer patients [32].

We have observed c-erbB2 overexpression in 24.3% of our patients, and only 5.6% of the examined tumors presented with level 3+. This observation does not differ considerably from other reports. Also, we did not observe any statistically significant correlation between protein overexpression and survival time or any other patho-clinical features of the neoplasmatic disease as is the case with other literature reports [30, 47-49]. Some authors indicate worse prognosis in cases with membranous c-erbB2 overexpression in colorectal tumors [6]. They support the hypothesis that *c-erbB2* up-regulation is associated with tumor progression and its metastatic phenotype, as they usually also observe chromosomal gains at the *c-erbB2* locus 17q in primary and secondary tumors. A tendency towards better survival has been observed in case of protein overexpression by a group of authors who had analyzed 156 colorectal cancer patients [49]. There also exist literature reports indicating worse prognosis in case of c-erbB2 protein overexpression [6].

Some authors report a positive correlation between c-erbB2 and better tumor differentiation despite differences in methodology, nevertheless without any correlation to survival time or other pathoclinical features [30, 44]. In their opinion c-erbB2 may contribute to tumor morphogenesis and tubular structures.

Many studies support the theory of colorectal cancer following an adenoma – carcinoma sequence [2, 31]. Alterations in the *p53* gene, the so called "guardian of the genome" play a very important part in this process [2, 18, 19, 31]. They usually result in mutated p53 protein production and accumulation [5, 31]. Although there is no agreement as to the prognostic role of p53 in colorectal cancer, a majority of reports indicate its negative association with prognosis [20, 22, 31]. The combination of data on p53 expression combined with data concerning other potentially prognostic factors may enhance the probability of correct prognosis [21]. The simultaneous existence of the *p53* mutation and the *c-erbB2* amplification possesses independent negative prognostic value in breast cancer patients without metastases to regional lymph nodes [27]. A statistically significant correlation has been observed between p53 and c-erbB2 expression in gastric cancer [25]. The coexistence of both these alterations is associated with more aggressive tumor behavior, although the authors of this particular paper have failed to provide some kind of explanation of this phenomenon. Other authors have performed evaluations of the immunohistochemical expression of five different proteins in resectable colorectal cancer: nm23, p53, c-erbB2, u-PA and VEGF [7]. p53 tended to correlate with liver metastases, and c-erbB2 did not show any inter-dependences. No correlations between p53 and c-erbB2 proteins were observed in the course of a study of 156 radically treated colorectal tumors, and p53 tended to correlate with worse prognosis [49]. We found p53 positive expression in 57.9% of tumors. We did not find any statistically significant correlations, neither between the mutual expression of the two investigated proteins nor between their expression and patient survival or in relation to other patho-clinical data, such as tumor location, WHO tumor type, depth of bowel wall infiltration, regional lymph node involvement, distant metastases, tumor differentiation and patient age and gender. We observed minor tendencies towards more frequent mutual c-erbB2 overexpression and p53 in younger patients ($p=0.07$), in case of mucinous adeno-carcinoma ($p=0.08$), and in cases of rectal localisation of the tumor ($p=0.06$) (Table I). We assume that the lack of any correlations in our study may be associated with a rather small number of tumors exhibiting c-erbB2 overexpression in our patient material.

Contrary to breast cancer cells, in the case of colorectal cancer cells cytoplasmic c-erbB2 is, predominantly, present [42-44, 48]. It appears in as many as 68.5% to 87% of colorectal tumors. The estimated prognostic value of cytoplasmic c-erbB2 in colorectal cancer patients varies distinctly from the negative to the positive [42, 48].

The different mechanisms leading to c-erbB2 accumulation in colorectal and breast cancers, and the diversity of cellular protein distribution suggest the different role of this protein in the biology of these two malignancies. It is necessary to unify the methodology of c-erbB2 protein expression in colorectal cancer cells, which could help to enroll larger patient groups with the overexpression of this particular protein and, thus, allow to perform reliable statistics on its prognostic role. We did not reveal any prognostic value of membranous c-erbB2 overexpression. The evaluation of p53 expression did not contribute to a more adequate estimation of prognosis in the investigated group of patients.

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