Original paper

A five-year follow up study of stage I-IV of rectal cancer with an emphasis on epidermal growth factor over-expression

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The key pro-proliferative pathway, based on EGFR-KRAS/BRAF-myc, is seen as the main goal of personalized therapy in rectal cancer. The objective of the study is to assess the EGFR immunoreactivity in rectal cancer and to estimate its relationship with the clinical outcome, especially as a predictor of poor outcomes.
Patients: applying exclusion criteria, 102 patients with stage I-IV rectal cancer, who had undergone scheduled surgery during the period 2005-2011, were included in the study. There was a follow-up study with a span of 5 years from the date of the surgery.
Immunohistochemistry using EGFR (EGFR Ab10, Clone111.6) was performed to detect an overexpression of the targeted receptor. Digital analysis of positive reactions of membranes was performed utilizing Visiopharm™.
The degree of EGFR intensity (log OR 0.854, OR 2.35, 95% CI: 1.14–4.85, $p = 0.021$) is a significant factor in the prognosis of death within 2 years of surgery. The OS curve showed a significant decrease after 40 months from the date of surgery in the cases where EGFR had a high expression. The ROC curve for the cancer stage, according to the UICC classification and EGFR expression, in order to predict a 2-year RFS, reached a high specificity value (ROC = 0.81, $p = 0.0408$).
Immunohistochemical EGFR expression is inexpensive, specific and broadly available.

Key words: EGFR, rectal cancer, follow up, immunohistochemistry.

Introduction

Epidermal growth factor (EGF) contributes to the genesis and progression of many malignancies, including rectal cancer. Under normal circumstances, EGF stimulates the proliferation of both mature epithelial cells and stem cells to renew the damaged epithelium. Nevertheless, if uncontrolled, it can lead to cancer [1, 2, 3]. The EGFR receptor specific for epidermal growth factor cells (c-ErbB-1/HER), belongs to transmembrane class 1 receptors containing two cysteine-rich domains in the extracellular part, and in the intra-plasmatic part a domain showing activity of tyrosine kinase activity [4, 5]. The binding of EGF or another ligand to EGFR causes phosphorylation and conformational change with tyrosine kinase activation in the endoplasmic domain. It is believed that the pathway, via the RAS protein family, is the key factor in initiating the process of cell

proliferation. To date, apart from EGF, a number of EGFR ligands have been described, including the following: transforming growth factor-α (TGF-α), amphiregulin (AR), heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), Cripto-1, schwannoma-derived growth factor (SDGF), vaccinia growth factor (VGF), spitz, and lin-3 [6, 7, 8]. The normal bowel mucosa does not express EGFR at a detectable level for immunohistochemistry (IHC). Any type of IHC expression proves the mutation and amplification of the *EGFR* gene and over-expression of that signaling pathway. In the case of rectal cancer, the EGFR over-expression is found in approximately 25-82% of cases. More interestingly, the intensity of EGFR expression correlates with IHC intensity, the severity of illness and the risk of distant metastases [9]. EGFR triggers secondary transmitters, including KRAS, BRAF and MYC, whilst simultaneously activating the PI3K pro-metabolic pathway [10]. Moreover, a parallel target is the MAPK pathway influencing cell survival. The occurrence of a mutation in the *KRAS* or *BRAF* gene means that these proteins are constantly active regardless of the activation or non-activation of EGFR. Currently, there is solid evidence that the mutations in the *KRAS* or *BRAF* gene are a negative predictor of response to the target therapy with the use of monoclonal antibodies against EGRF [10, 11].

Aim of the study

The objective of the study was to assess EGFR immunoreactivity in rectal cancer and to determine the clinical outcome, especially as a predictor of poor outcome.

Material and methods

The research was conducted in the Clinic of General, Oncological and Endocrine Surgery of the Provincial Hospital in Kielce, in the Department of Clinical and Experimental Pathomorphology of the *Collegium Medicum* of the Jan Kochanowski University in Kielce and in the Holy Cross Cancer Centre.

Study population

Using the appropriate inclusion and exclusion criteria, 102 patients with stage I-IV rectal cancer, who had been operated on between 2005-2011, qualified for the study. Inclusion criteria included male and female patients who had undergone scheduled surgery due to cancer of the rectum, in whom metastatic cancer was excluded and who did not have other gastrointestinal tract tumors. These patients presumably qualified for surgery with the intention of being treated, and otherwise suffered from no medical conditions (internal, cardiological, pulmonary). The clinical outcomes were overall survival (OS) and relapse-free survival (RFS) ,which was understood as local recurrence-free survival and dissemination-free survival.

Research methodology

The research methodology was divided into 2 stages. The first stage included a retrospective analysis of the medical history of patients who qualified for the study. The second stage included immunohistochemical studies assessing EGFR immunoreactivity in postoperative specimens. The study included patients without pre-operative radiotherapy. Finally, 102 patients, aged 41-87 years, were enrolled in the study. These included 41 women and 61 men with rectal cancer confirmed by a histopathological examination. There was a follow-up study with a span of 5 years from the date of the surgery. All the patients with adjuvant pre-operative radiotherapy were excluded, and all the participants in stage III-IV rectal cancer were subjected to FOLFOX-4 based chemotherapy (oxaliplatin 85 mg/m^2, calcium folinate 200 mg/m^2, fluorouracil 400 mg/m^2, fluorouracil 600 mg/m^2). The patients' written consent was obtained.

Immunohistochemical tests

Classic immunohistochemical tests, using an anti-EGFR antibody (EGFR 96 Ab10, clone 111.6), were performed. All the tests performed had been fully validated with the intention of *in vitro* use. All the reactions were carried out using BenchMark XT (Ventana Medical Systems; Roche Group, Tucson, USA). After a fully automated dewaxing and a repeated hydration reaction of the samples, the processes of unmasking of the antigen by proteinase K (37°C, 5 minutes) were conducted, followed by an incubation period with the primary antibodies (1 : 50 dilution, 20 minutes incubation). The time and the temperature of both the antigen retrieval and the incubation of primary antibodies were strictly in accordance with the manufacturer's recommendations, followed by further routine steps. A universal DAB Ventana detection kit was used. A 4-level scale describing the EGFR reactivity was used: 0 – no reaction, 1 – weak reaction, 2 – moderate reaction and 3 – strong reaction.

Digital and statistical analysis

All calculations were made using a digital slide analysis using a Hamammatsu NanoZoomer S210 slide scanner (Hamammatsu®, Hamamatsu City, Shizuoka Pref. Japan). After the scanning of the entire slide, a digital image analysis was then performed using the Visiopharm membrane application (Visiopharm®, Hoersholm, Denmark). The application used allowed us to diversify the intensity of the

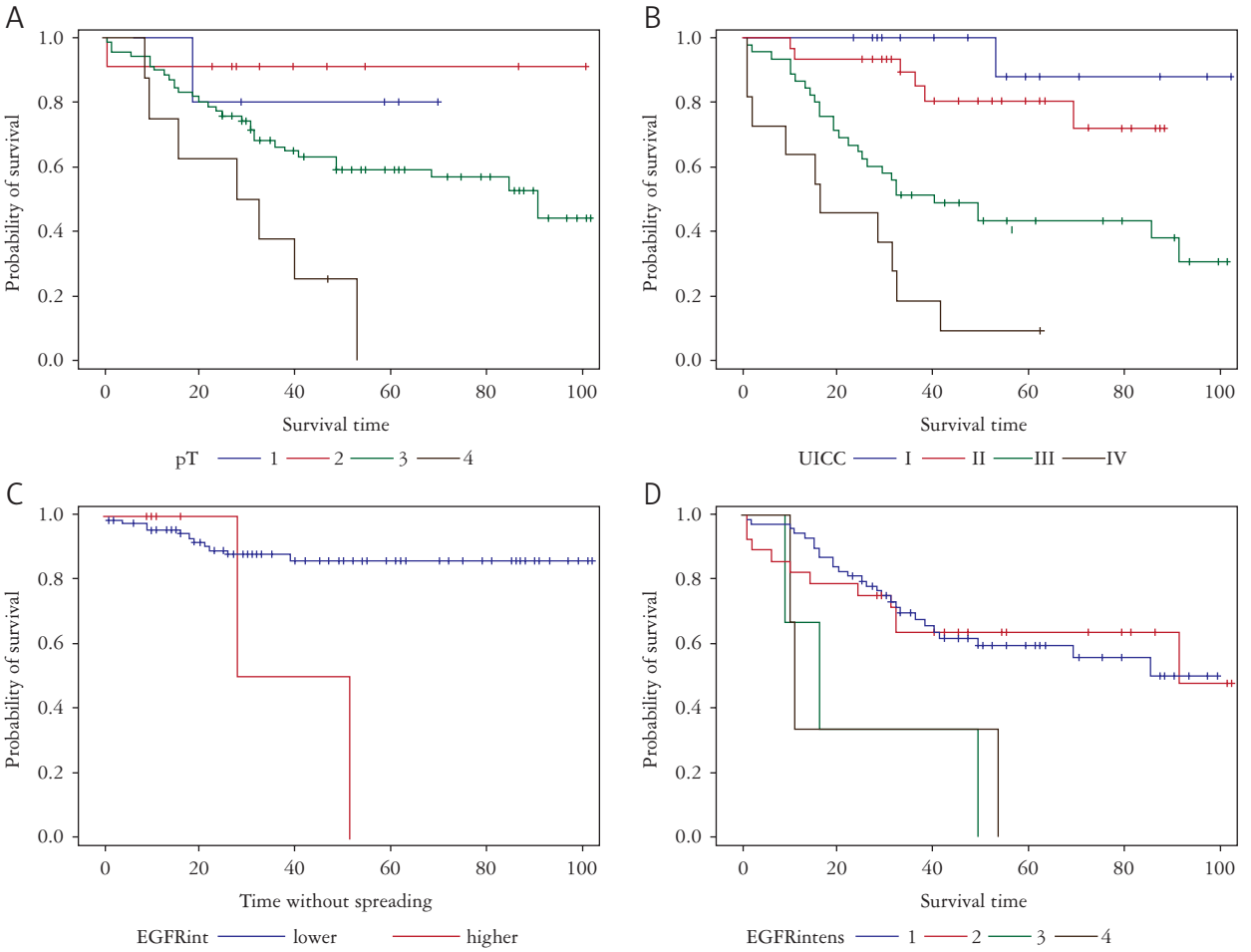

Fig. 1. OS curves in log-rank test: A) according pT p = 0.0001; B) according UICC stage p = 0.0001; C) according low EGFR vs. high EGFR expression p = 0.0297 (low intensity 0-1, high 2-3); D) overall survival (expressed in months) for patients according EGFR expression p = 0.0056

plasmalemmal reaction and avoid subjectivity. The collected data were subjected to statistical analysis using the Kaplan-Meier method, log-rank test, Cox proportional hazard model and logistic regression. In the tests, the significance level of 00.5 was adopted for the purposes of statistical inference. In statistical analyzes, licensed SAS 9.3 software and Excel were used.

Results

Follow up

The follow-up study covered a period of 5 years. The clinical outcomes were: overall survival and relapse-free survival understood as local recurrence-free survival and dissemination-free survival. Of the 102 patients studied, 56 survived until the end of observation period (i.e. 55%), including one with local recurrence and one with disseminated neoplastic process. Forty-six patients did not survive, among whom, 7 had local recurrence and the median survival was 26.6 months, while the median tumor-free survival was 21.6 months. Eighty-five per cent of women (F) and 92% of men (M) survived one year; 78% of women and 85% of men survived 2 years and 66% of women and 67% of men survived 3 years.

Relationship between EGFR immunoexpression and clinical-pathological features

The highest EGFR expression, at level 3, was determined in 3 patients, i.e. 2.9%; in 3 patients the EGFR expression was marked at level 2, and in 28 patients, i.e. 27.5%, at level 1. In as many as 68 patients, i.e. 66.7%, the EGFR expression was not detected. The study group was dominated by patients with pT3 tumor trait, whose EGFR expression was at level 0 or 1 (no expression). In contrast, patients with the highest EGFR expression at level 3, were patients with pT4 tumor. A higher grade of malignancy – G3 – is accompanied by a higher than level 3 expression of EGFR. Among patients with a higher level of EGFR (3), patients with the pN4 trait predominate. There is no statistically significant relationship

Table I. The comparison of 2-years RFC with predictive factors (a multistep logistic regression test)

Parameter		OR	95% CI	p
BMI	–0.0945	0.910	0.805-1.028	0.1297
Age	0.0133	0.013	0.961-1.069	0.6228
EGFR expression	0.854	0.35	1.14-4.85	0.021
UICC stage	1.596	0.93	2.15-11.35	0.0002

between EGFR expression and the histopathological type of cancer ($p = 0.1965$).

Outcome

As shown in Fig. 1, the OS curves decreased significantly, which is not surprising when pT or cancer stages were advanced, but graphs C and D show a poor result when EGFR was significantly overactive. Cancer spread or a patient's death occurred on average, close to 50 months after the surgery. The log-rank test supports the supposition that survival curves significantly differ statistically ($p = 0.0004$). The RFS probability was significantly higher in patients with low EGFR immunoreactivity than in patients with high EGFR immunoreactivity ($p = 0.0408$ in the log-rank test).

Multifactorial logistic regression results indicate that the degree of EGFR intensity (log OR = 0.854, OR = 2.35, 95% CI: 1.14-4.85, $p = 0.021$) is an important factor in the prediction of death within 2 years of surgery. With the increase in EGFR intensity by one degree, the chances of death by the end of the 2-year period are doubly increased (odds ratio OR = 2.35). Not surprisingly, the stage of cancer, according to the UICC classification, was a good prognosis of survival (log OR = 1.596, OR = 4.93, 95% CI: 2.15-11.35, $p = 0.0002$; Table I). The course of the logistic curve indicates that as the degree of EGFR intensity increases, the patient's probability of dying within 2 years of surgery increases (up to nearly 70% in the cases with strong EGFR expression; Fig. 2).

The stage of cancer has a statistically significant effect on the risk of a patient's death at $p > 0.0007$. The higher the stage of cancer , the risk of death increases by 10% (HR = 1.100). In summary of the BMI multistep selection, regarding patients' age and clinical stage, patients with a lower BMI appear to have a worse prognosis. More advanced cases had a higher risk of death (HR = 0.927). In contrast to this, the age of the patients at the time of diagnosis revealed a greater risk of death, by approximately 4%, with each subsequent decade (HR = 1.042). Similarly, The risk of death was found to increase by 64% with each increase in the UICC stage (HR = 1.641). The multivariable analysis shows that BMI does not significantly affect the risk of tumor dissemination ($p > 0.88735$).

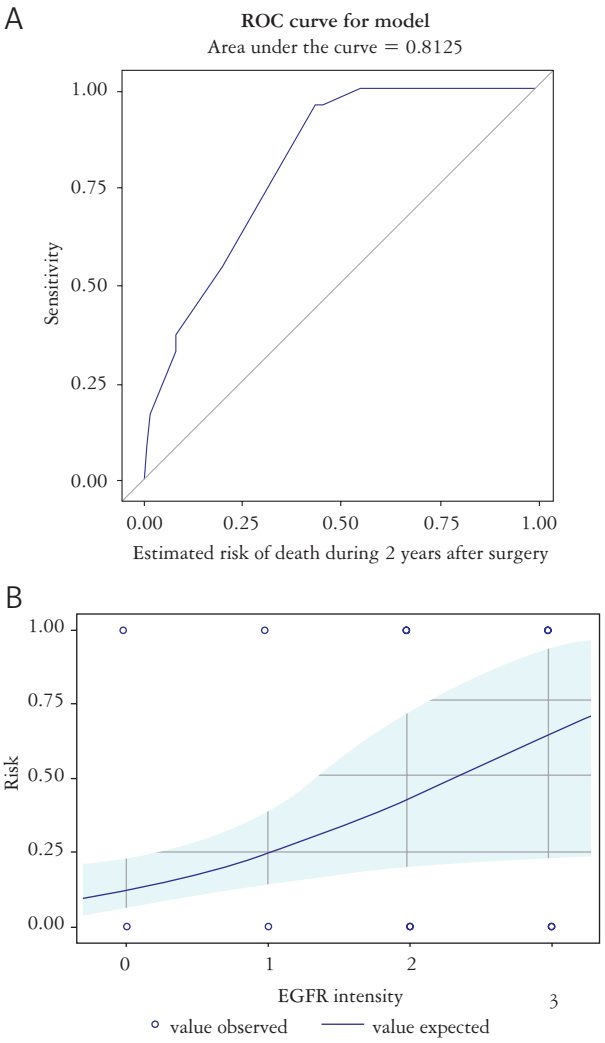

Fig. 2. ROC curve in UICC stage and EGFR expression for predict 2-years RFS ($p = 0.0408$) and estimated risk of death during 2 years according EGFR

Table II shows that high EGFR expression increases the risk of death 4 times (HR = 4.228), the risk of metastasis 4.6 times (HR = 4.650), as well as increasing the risk of recurrence 4.3 times (HR = 4.340).

Discussion

Piton *et al*. analyzed the sensitivity and the specificity of KRAS and BRAF immunohistochemistry,

Table II. The Cox proportional-hazards model with an independent variable EGFR immunoreactivity and death, metastasis and recurrence of cancer as a phenomenon determining the clinical outcome

Parameter	log hazard ratio	χ^2	p	HR
EGFR vs. death	1.441	10.511	0.0012	4.228
EGFR vs. metastases	1.536	0.900	0.0483	4.650
EGFR vs. recurrence	1.467	0.508	0.0610	4.340

presenting high specificity of V600BRAF as opposed to KRAS [12]. We are presented with a basic question concerning the methodology, as well as the cost of first-line testing involved in molecular pathway screening. Although rectal cancer is often mosaic and heterogeneous, we currently have a wide range of tests available, which can be a good way to gain a first insight into the genetic profile [13, 14]. An increase in the EGFR expression was observed in tumors of different locations and was usually associated with a poor prognosis, increased risk of relapse and a shorter survival rate [15, 16, 17]. However, the reports on the effect of the EGFR hyper-expression on survival in rectal cancer are conclusive. Some results of clinical-pathological studies showed that EGFR expression is a negative prognostic factor [18, 19]. EGFR expression is observed to be at varying degrees in solid tumors. According to Herbst *et al*., an EGFR overexpression in rectal cancer occurs in 50-70% of cancer cases, while according to other researchers, in about 25-82% [2, 20]. In our study, the EGFR expression at levels 3, 2 and 1 was determined in 33 patients, i.e. 34.68%. The studies to date have not explicitly confirmed the relationship between EGFR expression and the survival of patients with rectal cancer [21], although the study by Mayer et al. showed that EGFR expression in more than 50% of cancer cells is a negative prognostic factor [6]. Moreover, an overexpression of EGFR (upregulation) is associated with more aggressive tumor growth, a poorer prognosis and a higher resistance to radiation. Therefore, it can potentially be a useful marker in predicting a full response to treatment [2, 19, 22]. The results obtained in our studies confirmed the above-mentioned results – the probability of the survival was higher in patients with a low EGFR intensity ($p = 0.0004$). The likelihood of RFS was much higher in patients with a low EGFR expression than in patients with a high EGFR expression ($p = 0.0297$). The multicausal analysis showed that high EGFR intensity increases the risk of death 4-fold (HR = 4.228) and that high EGFR intensity increases the risk of metastases 4.6 times (HR = 4.650). The multivariate analysis also showed that high EGFR intensity increases the risk of cancer recurrence 4.3 times (HR = 4.3). Interestingly, our study showed that among patients with high EGFR levels, patients with pT4 predominated ($p = 0.0003$), although some researchers claim that the correlation between the EGFR overexpression and clinical-pathological parameters is not important [2, 23, 24, 25]. According to them, this may be only an additional molecular phenomenon that causes poor test results. Our own research showed that there was no statistically significant relationship between EGFR expression and the type of histopathological cancer ($p = 0.1965$). Based on the results of clinical observations, EGFR expression was found to be an adverse prognostic factor. The EGFR blocked by the monoclonal antibody entails the inhibition of many biological signaling pathways, often causing poor test results [2, 3, 24].

Conclusions

1. High EGFR immunoreactivity increases the risk of death four times and increases the risk of cancer spread 4.6 times as well as increasing the risk of cancer recurrence 4.3 times.

2. Marking of EGFR immunoreactivity is important in the monitoring and treatment of patients with rectal cancer.

Availability of data and materials

Please contact the first author for data requests.

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The Authors declare no conflict of interest.

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