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K-RAS point mutation, and amplification of *C-MYC* and *C-ERBB2* in colon adenocarcinoma

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Abstract: The routine multidisciplinary management of colon cancer is based mainly on tumor staging, histology, grading and vascular invasion. In this approach, important individual information derived from molecular characteristics of the tumor may be missed, especially since significant heterogeneity of molecular aberrations in cancer cells has been observed, and recognition of every of relationships between them may be of value. *K-RAS*, *C-MYC* and *C-ERBB2* are protooncogenes taking part in carcinogenesis and tumor progression in the colon. They influence cell proliferation, differentiation and survival. *K-RAS* point mutation, as well as amplification of *C-MYC* and *C-ERBB2* were searched in 84 primary colon adenocarcinomas resected with curative intent. Multiplex polymerase-chain reaction and restriction fragment length polymorphism were performed to assess codon 12 *K-RAS* point mutation. Amplification of *C-MYC* and *C-ERBB2* genes was evaluated by densitometry after agarose gel separation of the respective multiplex PCR products. No relation was found among mutated and/or amplified genes, and between searched molecular aberrations and pathoclinical features. In multivariate analysis, nodal status appeared to be the only independent prognostic indicator. In colon adenocarcinoma, codon 12 *K-RAS* point mutation and amplification of *C-MYC* and *C-ERBB2* seem to occur independently in the process of tumor progression. Amplification of *C-ERBB2* tends to associate with more advanced stage of disease. Concomitant occurrence of codon 12 *K-RAS* mutation, *C-MYC* and *C-ERBB2* amplification was of no prognostic value in respect to survival.

Key words: *K-RAS* - *C-MYC* - *C-ERBB2* - Colon adenocarcinoma

Introduction

Colorectal cancer is one of the main causes of cancer death [16]. In spite of preoperative and perioperative diagnostics together with the pathomorphologic examination of specimens resected, the real prognosis in a particular patient remains still unknown [26, 27]. Recognition of all genetic aberrations responsible for primary tumor development and dissemination of disease is the potential tool in detailed diagnosis of real tumor aggressiveness [19, 37]. Intratumor genetic heterogeneity of colorectal adenocarcinoma makes doubtful treating chosen single genetic aberration as a prognostic factor in colorectal cancer [8]. Probably the alternative pathways in colorectal carcinogenesis are responsible for different course of disease in patients with pathoclinically similar colorectal tumors. Their recognition can facilitate the settlement of the correct prognosis and enable successful prophylaxis and treatment of colorectal cancer [19]. Aside mutation in *APC* gene, responsible for FAP (familial adenomatous polyposis)-associated carcinogenesis, as well as in sporadic colorectal cancer, aberrations in *K-RAS*, *C-MYC*, and *C-ERBB2* are among the most often occurring events in early steps of malignant transformation and in further cancer progression in colon and rectum [1, 15, 18, 19].

K-RAS gene is located on chromosome 12, and encodes G protein showing GTPase activity. It takes part in cell signal transduction, and in the control of cell survival, proliferation, and differentiation [1, 37]. *K-RAS* mutations are closely associated with impairing GTPase activity and leaving G protein in its active form, what results in uncontrolled and excessive cell proliferation and disturbances in their differentiation [1, 19]. Mutation in codon 12 of *K-RAS* gene is the most commonly observed [2, 4, 38]. Aberrations in *RAS* contribute

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Table 1. Sequences of PCR primers used in the study

Gene	Primer	Sequence
C-ERBB2	erb1	5'-CACCTGTGAGGCTTCGAAGCTGCAG-3'
C-ERBB2	erb2	5'-GGATATCCAGGAGGTGCAGGGCTAC-3'
TK*	tk1	5'-CTCTGGGAACAACTCTGGGATGAGG-3'
TK	tk2	5'-ACTCAGGTGGTCCCAGGAAGTGTGG-3'
C-MYC	myc1	5'-CTCGAATTCCTTCCAGATATCCTCGCTG-3'
C-MYC	myc2	5'-CACTGCGCGCTGCGCCAGGTTT-3'
TPA**	tpa1	5'-CGACAATGACATTGGTAAGAGCTCG-3'
TPA	tpa2	5'-ACTTACAGGCCTCATGCTTGCCGTA-3'
K-RAS	KR1	5'-ACTGAATATAAACTTGTGGTAGTTGGACCT-3'
K-RAS	KR2	5'-TCAAAGAATGGTCCTGGACC-3'

*TK - thymidyne kinase; **TPA - plasminogen tissue activator

to neoangiogenesis and to metastatic progression independently of proliferative hyperactivity of RAS proteins. Similar effect can be caused by C-ERBB2 overexpression [1, 32]. *C-ERBB2* gene is located on chromosome 17 and encodes the cell membrane $p185^{HER2}$ protein [3, 18]. It belongs to type I epithelial growth factor receptor family of cell surface receptors, and is responsible for signal transmission required for normal cell proliferation and differentiation [18, 30]. Among several *C-ERBB2* signaling targets is MAP kinase (mitogen activated protein) involved in proliferation and maturation of numerous cell systems. $p185^{HER2}$ and G proteins may be the factors of MAP-stimulated cascade of changes. One of their substrates is C-MYC protein [18]. In breast cancer, *C-ERBB2* amplification occurs in about 25% of cases [23], what is thought to lead to p185HER2 overexpression. Consecutive increase in HER2/neu kinase activity initiates signal transduction resulting in excessive cell proliferation [30]. C-ERBB2 protein overexpression in colorectal cancer cells does not always follow the amplification of *C-ERBB2* gene [29].

Protooncogene *C-MYC* is located on chromosome 8, and encodes the transcription factor responsible for cell proliferation promotion through cell-cycle reentry [17, 33]. This ability determines its contribution to carcinogenesis, especially in cases with aberrations in *C-MYC* gene. *CDK4* seems to be the target gene for the activity of C-MYC closely connected with cell cycle regulation and tumorigenesis promotion [17]. Several reports indicate that genetic alterations of *C-MYC* oncogene play an important role in induction and progression of breast cancer [13, 34, 40]. Overexpression of C-MYC protein to some extent correlates with gene amplification but not all tumors with *C-MYC* amplification display increased level of this oncoprotein and some tumors reveal C-MYC overexpression without gene amplification [9, 13]. In colorectal primary tumors, amplification of *C-MYC* is less often observed than overexpression of C-MYC protein [24]. There was no correlation noticed between *C-MYC* amplification and the amount of C-MYC protein in primary colorectal cancer [33]. Amplification probably associates with increased potential of colorectal cancer to distal metastatic progression [22, 33].

In the present study, we analysed the occurrence and relationship among aberrations in protooncogenes involved in the process of tumor formation, and normally responsible for cell proliferation and differentiation. Since relations between overexpression of these genes are well recognized [15, 18, 19], *K-RAS* mutation in codon 12, as well as *C-MYC* and *C-ERBB2* amplification in the resected colon adenocarcinomas were examined. These molecular aberrations were then correlated with the following parameters: age, gender, tumor location (right or left colon), pTNM status, histopathology type according to WHO, tumor differentiation, and survival time.

Materials and methods

Patients. Eighty four patients with resectable colon adenocarcinoma were included. They were operated on with curative intent in five Gdańsk hospitals between February 1996 and February 1998. The patients, 39 females and 45 males were from 33 to 87 years old (mean 64 yrs, median 65 yrs). The minimal follow-up period for living patients (n=44) was 25 months, and the maximal period was 70 months (mean 59.5 months, median 61.5 months). For the patients who died (n=40) the minimal follow-up period was 4 months, and the maximal 58 months (mean 22.7 months, median 20 months). The median follow-up period for the whole group was 47 months.

Tissue preparation. Two sides of colon were considered; right, including caecum, ascending colon, hepatic flexure with proximal 2/3 of transverse colon, and left, including distal 1/3 of transverse colon, spleen flexure, descending colon and sigmoid colon. Immediately after surgery, specimens of tumor tissue were collected, frozen in the liquid nitrogen and stored at -80°C until examination at

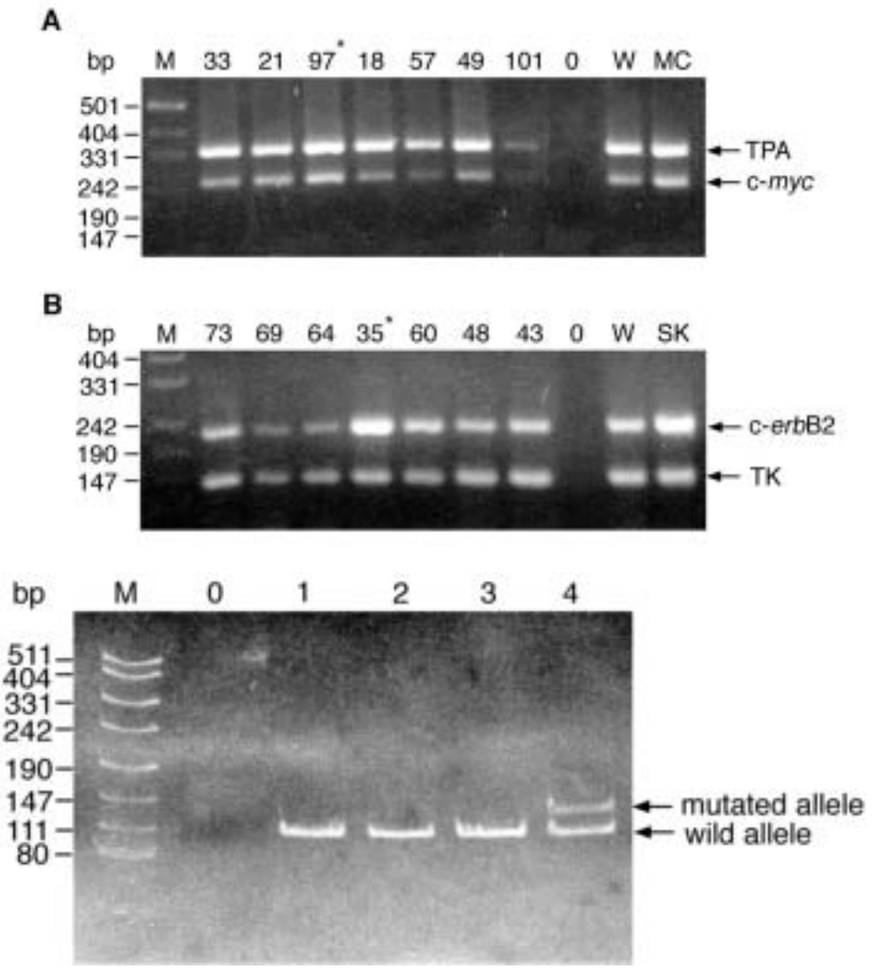

Fig. 1. Analysis of *C-MYC* and *C-ERBB2* amplification in colon adenocarcinoma. **A**: PCR analysis of *C-MYC* amplification with the gene for tissue plasminogen activator (TPA) as a reference gene. **B**: PCR analysis of *C-ERBB2* amplification with the gene for thymidine kinase (TK) as a reference gene. Lane M, DNA molecular weight marker; lane 0, product of control PCR performed without the template; lane W, PCR product with wild DNA isolated from healthy person; lane MC and SK, product of PCR performed with DNA isolated from cells with defined *C-MYC* (MCF 7) and *C-ERBB2* (SKBR 3) amplification, respectively. The numbers on the top indicate the patient's number. Tissues showing *C-MYC* or *C-ERBB2* amplification are marked by asterisk.

Fig. 2. Analysis of *K-RAS* point mutation in codon 12 in colon adenocarcinoma. A 157 bp fragment of *K-RAS* exon 1 was amplified and digested with BstN1 enzyme and separated on 8% polyacrylamide gel as described in Materials and methods. Lane M, DNA molecular weight marker; lane 1-4, the digested products of PCR performed on DNA isolated from examined tissues; lane 0, the digested products of PCR performed without the template (negative control of PCR reaction).

the Department of Molecular Biology, Clinical Biochemistry Division, Medical University of Gdańsk. The material was also assessed microscopically by two independent pathologists at the Department of Pathology, Medical University of Gdańsk.

DNA extraction. DNA extraction from the tissues examined was carried out using Genomic DNA Prep Plus kit (A&A Biotechnology, Poland). The DNA content was measured by light absorption at 260 nm. The purity of DNA was assessed based on the calculated ratio A_{260}/A_{280}.

Determination of *C-MYC* and *C-ERBB2* amplification. Amplification of *C-MYC* and *C-ERBB2* was assessed by semi-quantitative multiplex PCR assay, which in our hands proved to be useful in evaluating changes in a template copy number [13, 23]. The reaction mixture contained 50 mM Tris-HCl, pH 9.0, 20 mM ammonium sulfate, 100-500 ng of template, 0.50 μM each of 5' and 3' primers, 0.25 μM of each dNTP, 2.5 mM $MgCl_2$ and 1 Unit of Tfl DNA polymerase (Epicentre Technologies). The PCR reaction was performed for 30 cycles of 94˚C (1 min), 65˚C (1 min), 72˚C (2 min) and 94˚C (1 min), 56˚C (1 min), 72˚C (2 min) for *C-MYC* and *C-ERBB2* amplification, respectively. The amplification assessment of *C-MYC* and *C-ERBB2* was performed with the primers described by Kowara *et al.* [23]. Tissue plasminogen activator (TPA) and thymidine kinase (TK) were the reference genes for *C-MYC* and *C-ERBB2* amplification, respectively. The primers for TPA and TK amplification assessment were described by Lonn *et al.* [25]. The PCR products were separated by agarose gel electrophoresis and the ethidium bromide-stained bands were quantified with the use of Gel Doc 2000 system (Bio-Rad) and compared using computer software Quantity One (Bio-Rad). To produce titration curve of gene amplification, we used MCF7 and SKBR3 cells with defined *C-MYC* and *C-ERBB2* gene copy number, respectively. The representative patterns of multiplex PCR are presented in Figure 1. All primers used were from Integrated DNA Technologies, Inc. (Coralville, IA, USA) and are presented in Table 1.

Determination of *K-RAS* mutation. The point mutation of *K-RAS* gene in codon 12 was determined by PCR-RFLP. Amplification of the fragment of *K-RAS* exon 1 was performed with the KR1 and KR2 primers (Table 1). The KR1 primer has a restriction site for BstNI (underlined), which includes two first nucleotides in codon 12. Second restriction site for BstNI (underlined) is within KR2. The reaction mixture contained 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0 .001% gelatin, 100 ng of template, 0.50 μM each of 5' and 3' primers, 0.25 μM of each dNTP, and 0.4 Unit of Ampli Taq Gold DNA polymerase (Perkin Elmer, Roche, USA). The PCR reaction was performed for 30 cycles of 95˚C (45 s), 56˚C (30 s), 72˚C (30 s) and final extension at 72˚C for 10 min. Following amplification, 10 μl of the PCR products were digested for 1 hour by restriction enzyme BstNI (Sigma Bioscience, USA). Reaction products were separated on 8% polyacrylamide gel (Bio-Rad Labora-

tories, USA), stained with ethidium bromide and visualized using UV illuminator. The PCR product contains 157 bp. Digestion with restriction enzyme of the *K-RAS* fragment amplified on normal matrix results in obtaining 3 fragments; 14, 29 and 114 bp. Mutation in codon 12 results in the loss of one of the restriction sites and only two fragments, 14 and 143 bp, can be obtained (Fig. 2).

Statistics. Frequency tables and chi-square test were used to compare categorical variables ($p<0.01$ for statistically significant differences). Survival analysis was performed according to Kaplan-Meier method. Log-rank test and Cox proportional hazard model was used in univariate and multivariate analysis, respectively ($p<0.05$ for statistically significant differences).

Results

Median of survival time reached 58 months according to absolute survival curve. Table 2 contains data on frequency of *K-RAS* point mutation, *C-MYC* and *C-ERBB2* amplification in relation to each other, and to pTNM stage, feature pT, nodal status, tumor differentiation, cancer histological type according to WHO, and tumor location. The p values were based on chi-square test. *K-RAS* mutation in codon 12 was observed in 27 of 84 cases (32%). Amplification of *C-ERBB2* gene and *C-MYC* gene was observed in 16% (13 of 84) of examined specimens. In the analyzed group of colon adenocarcinomas, the occurrence of at least one of searched gene aberrations was observed in 46 tumors (55%), and lack of any aberrations in 38 (45%).

We did not observe statistically significant differences in the occurrence of examined parameters. The analysis showed only tendency for more frequent occurrence of *C-ERBB2* amplification in advanced tumors (p=0.06). *C-MYC* amplification occurred more often in left-sided cancers (7 out of 10; p=0.07). *C-MYC* amplification was observed in cases without mutant *K-RAS* (11 out of 13 amplified). Similarly, *C-ERBB2* amplification was also observed in tissues with no point mutation in *K-RAS* (10 out of 13 amplified). Conversely, *K-RAS* was mutated when no amplification in *C-ERBB2* (24 out of 27 mutated) or in *C-MYC* (25 out of 27 mutated) occurred. In only two cases amplification of *C-MYC* and *C-ERBB2* was diagnosed in the same tumor. In these cases, no *K-RAS* mutation was observed. No *C-MYC* amplification was noticed among mucinous type adenocarcinomas. In univariate and multivariate survival analysis according to Cox proportional hazard model, only nodal status turned out to be an independent prognostic factor in our study (p=0.039, Table 3).

Table 2. The occurrence of *K-RAS*, *C-MYC*, and *C-ERBB2* molecular abnormalities in relation to each other, to tumor stage, degree of differentiation, WHO type, and tumor location

Examined feature	*K-RAS* mutation		*C-ERBB2* amplification		*C-MYC* amplification	
	yes	no	yes	no	yes	no
C-ERBB2 amplification						
yes	3	10				
no	24	47				
	p=0.66					
C-MYC amplification						
yes	2	11	2	11		
no	25	46	11	60		
	p=0.28		p=0.68			
pTNM stage						
I	2	7	2	7	3	6
II	13	24	2	35	5	32
III	10	24	7	27	4	30
IV	2	2	2	2	1	3
	p=0.74		p=0.06		p=0.40	
T_{1-3}	9	28	5	32	6	31
T_4	18	29	8	39	7	40
	p=0.26		p=0.89		p=0.89	
N_0	15	34	8	41	8	41
N_{1+2}	12	23	5	30	5	30
	p=0.9		p=0.96		p=0.96	
Tumor differentiation						
good	12	21	6	27	6	27
moderate	14	29	6	37	6	37
poor	1	7	1	7	1	7
	p=0.43		p=0.85		p=0.85	
WHO type						
adenocarcinoma	25	51	12	64	13	63
adenocarcinoma mucinosum	2	6	1	7	0	8
	p=1.0		p=1.0		p=0.35	
Tumor location						
right	16	24	7	33	3	37
left	11	33	6	38	10	34
	p=0.22		p=0.85		p=0.07	

Discussion

Molecular and immunohistochemical studies show considerable heterogeneity of genetic aberrations in colorectal cancer [8, 19, 37]. Attention is paid to the possibility of colorectal cancer development on the basis of totally different genetic aberrations. Continuous evolution of tumor cells results in creation of neoplasms pathoclinically similar but possessing totally different genotype. While various theories of colorectal carcinogenesis and tumor progression are discussed, it should be emphasized that the validity of each of them in particular case could probably be demonstrated [19]. It seems that recognition of the sequence of genetic changes in primary tumor with known progression level can be a valuable advice in studies on treatment of colorectal cancer. The knowledge of early events in carcinogenesis and tumor progression is of particular value. The aberrations in *APC* suppressor gene controlling β-catenin activity and in *CTNNB1* gene encoding β-catenin are thought to play an important role in tumo-

Table 3. Results of log rank test and multivariate analysis of the investigated features

Feature	Log rank	Multivariate analysis
N0 vs. N1+2	p=0.02	p=0.039
T1-3 vs. T4	p=0.11	p=0.59
M0 vs. M1	p=0.13	p=0.19
Age below and above median	p=0.13	p=0.27
C-MYC amplified vs. nonamplified	p=0.63	p=0.67
C-ERBB2 amplified vs. nonamplified	p=0.71	p=0.88
K-RAS mutated vs. nonmutated	p=0.95	p=0.44

rigenesis promotion in colorectum [15, 41]. β-catenin, which is present in almost all steps and in every aspect of colorectal carcinogenesis, is thought to be one of the most important molecules in the process of tumorigenesis [3, 41]. RAS and ERBB2 are the prooncogenic factors which release β-catenin, and/or facilitate its translocation into the cell nucleus [3]. The combination of TCF-4 with β-catenin contributes to expression of C-MYC that takes part in carcinogenesis [3, 41]. The loss of control on β-catenin can contribute to activation of *K-RAS* by point mutation [3, 15]. *K-RAS*, *C-ERBB2* and *C-MYC* genes normally are responsible for proper cell proliferation, differentiation, and survival [1, 10, 18]. Aberration of their physiological function contributes to carcinogenesis and tumor progression, also in early phases of these events [3, 15, 41].

In our study, only adenocarcinomas located in the colon were examined, and tumors of rectosigmoid and rectal regions were excluded from the investigation. They were mostly advanced cancers (Table 2). The material was estimated prospectively. In every case resection with curative intent was performed. Among patients with liver metastases, only those with a chance for cure after liver metastases resection were included in the study. The frequency of the examined aberrations corresponded with that observed by other authors [1, 2, 5, 11]. It is worth noticing that there was no correlation between amplification of *C-MYC* and *C-ERBB2* on the one hand and *K-RAS* point mutation on the other. It should be stressed that in 45% of primary tumors (38 cases) none of the examined genetic aberrations was observed. Our results seem to support the theory according to which accumulation of genetic changes does not necessarily occur during tumor progression.

In the study of Zhang *et al.* [42], changes in *K-RAS*, and *C-ERBB2* genes correlated with histological type of colorectal cancer: more frequent *K-RAS* mutations were associated with mucinous type of tumors. In spite of similar percentages of mucinous tumor type in their series (11%) and in our material (10%), we were not able to confirm these associations. The large number of patients in the former study and thus the ability to detect smaller differences could partially account for this discrepancy.

The prognostic value of *K-RAS* mutation in colorectal cancer remains controversial. This abnormality was described to correlate with distant spread and worse survival [5, 12, 38]. The negative prognostic value of *K-RAS* mutation was found in 117 colorectal cancer patients with over 10 years of follow-up [38] as well as in 98 colorectal cancer patients with median follow-up of 21 months [12]. In both studies, codon 12 and 13 mutations were analysed. In another study including 229 patients with colon and sigmo-rectal cancer with 7 years of follow-up, negative influence of *K-RAS* mutation on survival was confined to stage II and not to stage III of the disease [2]. In the latter work, two thirds of *K-RAS* mutations were found in codon 12, and *K-RAS* mutated tumors were more frequently observed in patients with more than 3 metastatic regional lymph nodes. Andreyev *et al.* [5] collected data on 3439 patients with colorectal cancer and found prognostically independent association between only one mutation in codon 12 of *K-RAS* (glycine to valine) and increased risk of disease recurrence and death in patients with Dukes C. They did not observe such an association in Dukes B patients. In the present report based on 84 colon adenocarcinomas in a group of patients with median follow up of 47 months, occurrence of *K-RAS* mutation in codon 12 neither affected survival nor was associated with clinical or pathological variables (Table 2). Similar observations were reported by other authors [4, 6, 24, 28, 39].

The finding that *C-ERBB2* gene overexpression in breast cancer associates with worse prognosis became the beginning for studies on the role of *C-ERBB2* in other neoplasms [14, 18, 21]. It was demonstrated that *C-ERBB2* overexpression increased metastatic potential of malignant cells in some neoplasms, and that it played a role in resistance to some cytostatics [18]. In the study involving breast cancer patients, it was demonstrated that *C-ERBB2* overexpression is mainly dependent upon amplification of this gene [35]. Press and co-workers [31] showed that *C-ERBB2* amplification negatively influenced prognosis of women with breast cancer. Osako *et al*. presented immunohistochemical analysis of 146 colorectal tumors, and showed that overexpression of C-ERBB2 protein occurred to be an independent indicator of poor prognosis [29]. In their study they found amplification of *C-ERBB2* in only 2 out of 44 colorectal cancer cases. According to other authors, C-ERBB2 protein overexpression correlates negatively with disease-free survival [20]. In our material, a trend towards an association with advanced stage and no other relationship to clinical or pathological parameters was found for *C-ERBB2* amplification (Table 2).

Sikora *et al.* [36] did not observe amplification of *C-MYC* in any from 15 colorectal cancers, finding mRNA and C-MYC protein in 12 out of 15 investigated tumors. According to that study, in patients with liver metastases and with C-MYC protein overexpression in cancer cells of primary tumors, C-MYC protein overexpression was also noticed in metastatic foci. In the study of Kozma *et al.* [22], *C-MYC* amplification was associated with a high risk of distant but not regional spread. In our material, only 4 patients relapsed at distant sites (solitary resectable liver metastases) and thus an appropriate analysis of dissemination predictors was impossible. Higher frequency of *C-MYC* amplification in colorectal cancer metastases in comparison to primary tumors indicates that the clones of cells harbouring this abnormality may be selected in the process of dissemination [33]. According to the cited study, overexpression of C-MYC protein in metastases did not correlate in any way with *C-MYC* gene copy number. The authors concluded that amplification of *C-MYC* was related to cancer metastatic progression, and that mechanisms responsible for gene amplification differed from those responsible for protein overexpression. Augenlicht and co-workers [7] found no prognostic impact of *C-MYC* amplification in colon cancer. However, they described a positive predictive value of this abnormality for the results of chemotherapy in Duke's stages B2 and C. In their series, *C-MYC* amplification was associated with high tumor grade. According to other authors, *C-MYC* amplification is linked to poor prognosis [22, 23]. On the other hand, in a study of Masramon *et al.* no association of *C-MYC* amplification with clinical or pathological variables was found except a high correlation with tumor stage and a trend towards higher frequency of this abnormality in left-sided tumors [24]. In our study we did not find any correlation between *C-MYC* amplification and the studied variables in spite of tendency to more frequent occurrence of *C-MYC* amplification in left-sided colon tumors (p=0.07, Table 2). In a study of Rochlitz *et al.* [33], and Masramon *et al.* [24], neither *C-MYC* status nor C-MYC expression level correlated with *K-RAS* mutation.

Our results indicate that in colon adenocarcinoma, mutation in codon 12 of *K-RAS* and amplification of *C-ERBB2* and *C-MYC* seem to occur independently in the process of tumor progression. Amplification of *C-ERBB2* tends to associate with more advanced stage of the disease. Examination of concomitant codon 12 *K-RAS* mutation, *C-MYC* and *C-ERBB2* amplification was of no prognostic value in respect to survival. According to multivariate analysis, nodal status appeared to be the only prognostic indicator for survival.

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