PREDICTIVE AND PROGNOSTIC FACTORS IN THE COMPLEX TREATMENT OF PATIENTS WITH COLORECTAL CANCER

László Landherr

University of Medicine and Pharmacy Tg. Mures, School of PhD Studies, Tg. Mures, Romania

Colon cancer is the second most prevalent lethal cancer. The main cause for high mortality rate is that the prognosis for progressed metastatic colon cancer is most unfavorable. Recent data suggest that disease outcome can be further improved by the addition of targeted biological agents to the first- or second-line treatment. As a result of molecularly targeted anti-EGFR therapies (cetuximab and panitumumab) complementing chemotherapy, liver metastases can reduce in size and become operable in certain patients, which can contribute to the complete recovery of the patient. The main problem, however, is the fact that a positive response only occurs in one third of the patients, even in the case of chemotherapy combined protocol, and the side effects are considerable. For the application of individually tailored treatments, it is an urgent need to develop a system of biomarkers that can predict the effect of treatment and provide information about the optimal selection of both chemotherapy and biological treatment. It should be clarified what the most important requirements of a good and reliable biomarker are. As currently there is no precise predictive molecular diagnostics at our disposal, oncologists have to make one of two choices: they treat a large number of patients with anti-EGFR agents which has negative effects on the quality of life and also reduces the patient's chances of getting appropriate treatment or, if the oncologists refuse to take risks, they omit the use of anti-EGFR treatment in which case those patients for whom this would have been the appropriate treatment are also denied the chance of short-term survival or recovery. Clinical data (response rate, time to progression (TTP) and overall survival (OS)) of 130 colorectal cancer patients have been retrospectively analyzed. Patients have received different chemotherapy protocols in combination with anti-VEGF antibody or with anti-EGFR antibody therapies. EGFR expression was evaluated with immunohistochemistry, KRAS, BRAF and PIK3CA mutations were evaluated by direct sequencing and high resolution melting analysis in the archived formalin-fixed, paraffin-embedded tissue samples. The study found similar efficacy of first-line therapeutic protocols. Protocols combining chemotherapy with biological therapies achieved better overall survival but this difference was not significant (OS: 35.9 versus 36.7 months). The frequency of KRAS mutations was 44% (n = 100). None of the KRAS mutant tumors responded to the anti-EGFR monotherapy. TTP in the case of cetuximab monotherapy was twice longer (208 months) than in the KRAS mutant tumors (104 months). One BRAF mutant tumor was also identified (4%) This tumor was also resistant to cetuximab monotherapy. The KRAS and BRAF mutations excluded each other. Except one case, the KRAS status was identical in both the primary tumor and the metastasis. In contrast, PIK3CA mutations were heterogeneous in different tumor samples. In 5 out of 6 cases the mutation status of PIK3CA was different in the primary tumor and the metastasis. New biological therapies provide an additional clinical benefit only for a subset of patients. We need biomarkers to identify these patients. KRAS and most probably BRAF testing can double the efficacy of the anti-EGFR therapies, but we need additional molecular diagnostic tests. PIK3CA is an important candidate but we might need to take biopsy directly from the metastasis or we have to evaluate the circulating tumor cells to judge the molecular status of distant metastasis. Hungarian Oncology 54: 383–394, 2010

Keywords: predictive, prognostic, chemotherapy, targeted therapy, biomarker

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Levelezési cím:
Dr. Landherr László
Fővárosi Uzsoki utcai Kórház
Onkoradiológiai Osztály
1145 Budapest
Uzsoki u. 29.
Tel./Fax:
(36-1) 467-3776
E-mail:
landherr@uzsoki.hu

INTRODUCTION

Colorectal cancer (CRC) is the second most prevalent malignancy in developed countries, following lung cancer in men and breast cancer in women. Its incidence is increasing slowly. Globally, the number of new CRC patients is over 1 million per year, and CRC leads to approximately 500,000 deaths yearly. In the United States CRC is the second most common cause of death. It is estimated that in 2008 nearly 150,000 new cases were diagnosed and the disease accounted for 50,000 deaths. In 2006 alone, 412,900 colorectal cancer cases and 207,400

CRC deaths were registered in Europe. It is often considered a consequence of the aging population, as CRC mostly affects the elderly. Forty percent of the tumors are diagnosed over 75 and the incidence is increasing with age, doubling every 7 years over 50. According to a survey by the SEER (Surveillance, Epidemiology, and End Results) database in the US, colon cancer is the leading cause of death among the insured aged 75 to 84. Approximately 9000 new cases and 5000 CRC deaths are expected in Hungary yearly. One reason for the high mortality rate is that at the time of diagnosis most colorectal tumors have already invaded the bowel wall: 36% of the patients have locally advanced disease (Dukes C, stage III), and 20% already have distant metastases (stage IV). Chemotherapy for stage IV metastatic colorectal cancer has improved significantly in recent years. In addition to the standard 5-fluorouracil/folinate (5-FU/FA) treatments, new drugs were introduced (oxaliplatin, irinotecan), and most recently the „targeted" biological therapeutic agents (bevacizumab, cetuximab, panitumumab) have also proved efficient. The increasingly efficient and therefore increasingly extended treatment of this large patient population with innovative targeted biological agents puts a heavy financial load on the society. Hence it is of great importance that the expensive therapies are only received by those patients whose disease is likely to respond. The explosive development of molecular pathology reveals more and more details of the intracellular signal transduction mechanisms, providing clinicians with novel predictive markers for treatment efficacy. The physician's role is to verify the predictive value of these in practice.

AIMS OF THE THESIS

- Comparison of clinical responses (response rate, overall survival (OS) and time to progression (TTP) to different treatment protocols with or without biological targeted therapies.
- Clinical validation of the predictive diagnostic value of KRAS mutation testing for individualized therapy of colorectal patients with EGFR inhibitors.
- Investigation of the predictive value of BRAF and PIK3CA mutation testing for individualized therapy of colorectal patients with EGFR inhibitors.
- The aim of these theses was to evaluate the efficacy of chemotherapeutic protocols available for the treatment of metastatic colorectal cancer patients. The second aim was to validate and investigate the value of predictive biomarkers in the personalized use of anti-EGFR (epidermal growth factor receptor) therapies.

METHODS

Clinical data of 130 histologically verified metastatic colorectal patients were retrospectively analyzed. Patients were treated with chemotherapy and biological targeted therapies between 01.08.1994. and 01.04.2009. at the Outpatient Clinic of Uzsoki Municipal Hospital, Budapest, Hungary. During this period unified guidelines were not available for the treatment of colorectal cancer; therefore these patients received various treatment protocols which provide special opportunity to compare the clinical efficacy of different therapies.

Tumor samples of patients treated with the anti-EGFR antibody cetuximab were not selected by KRAS testing, only by immunohistochemical positivity for EGFR. This provides an excellent opportunity to validate the predictive diagnostic value of KRAS testing.

In cases of distant metastasis or relapse, histological examination (cytology, core-biopsy or surgical specimen) was also performed from the additional tumor sites. Therefore, it is possible to study the correlation between the molecular status of multiple tumor sites within the same patient, and the clinical response to anti-EGFR therapy.

The follow-up of the patients was done by CT or MRI examinations every 6–10 weeks. The therapeutic response was evaluated by the Response Evaluation Criteria in Solid Tumors (RECIST). In case of obvious progression indicated by the clinical deterioration of patients, the horizontal imaging was abandoned.

DNA was extracted from 3–5 sections of 5 mm thickness. The sections were washed with 100% ethanol, and digested with 5 mg/ml proteinase K for 64 h at 50°C. Protein was precipitated with 3.3 M ammonium acetate, the supernatant was washed with isopropanol containing 0.1 mg/ml glycogen (1–2 h incubation at -20°C) and with 70% ethanol (-20°C). Complete removal of the alcohol was followed by dissolution of the DNA.

After DNA extraction exon 2 of the KRAS gene and exon 15 of the BRAF gene were amplified using nested PCR with „touch-down" protocol. Primers and the dNTPs were removed from the PCR products with ExoSAP IT. Sequencing was done with BigDye Terminator v3.1 Sequencing Kit (Applied Biosystems) and the sequencing fragments were detected via capillary electrophoresis using ABI Prism 3130 Genetic Analyzer (Applied Biosystems). High quality sequence variations were confirmed from both directions in two independent PCR reactions of the original DNA samples.

Primers were designed to span exon 9 and exon 20 of the PIK3CA gene. The intercalating dye used was Reso-Lite (Roche, Germany). The 20 µl reaction mixture was made up using 10 µl Roche LightCycler480 High Resolution Melting Master premix (Roche, Germany) and consisted of 25 ng of genomic DNA, 2 µl c = 25 mM $MgCl_2$, 0.5 µl c = 10 mM of each primer. All PCR reactions were performed in duplicate. PCR cycling and HRM analysis was performed on the LightCycler480 (Roche, Germany).

The expression of EGFR protein was determined by immunohistochemistry (IHC) using Dako EGFR PharmDx kits (DakoCytomation) or Ventana, clone:

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Table 1. Results of different treatment protocols

Type of treatment	N (out of 130)	%	TTP (month)	OS (month)	Still alive	Lost to follow-up
First line						
Oxaliplatin-based	54	41.5	9.3	32.7	17	10
Irinotecan-based	59	45.4	10.7	32.1	24	21
Bevacizumab + chemo	43	33.1	10.2	45.8	24	15
Bevacizumab + FOLFOX	10	7.7	10.0	–	10	0
Bevacizumab + FOLFIRI	33	25.4	10.2	45.8	14	15
Cetuximab + FOLFIRI	10	7.7	10.0	25.2	3	2
Second line						
Oxaliplatin-based	24	18.5	8.1	28.8	8	6.0
Irinotecan-based	46	35.4	6.6	38.4	14	12
Bevacizumab + chemo	19	14.6	5.8	26.3	11	3
Cetuximab + chemo	4	3.1	10.1	44.3	1	1
Bevacizumab + FOLFOX	1	0.8	–	–	1	0.0
Bevacizumab + FOLFIRI	18	13.8	5.8	26.3	10	3.0
Cetuximab + FOLFIRI	3	2.3	12.4	25.8	1	1
Third line or more						
Oxaliplatin-based	13	10.0	–	–	–	–
Irinotecan-based	15	11.5	–	–	–	–
Bevacizumab + chemo	7	5.4	–	–	–	–
Cetuximab + chemo	7	5.4	–	–	–	–
Bevacizumab + FOLFOX	0	0.0	–	–	–	–
Bevacizumab + FOLFIRI	7	5.4	–	–	–	–
Cetuximab + FOLFIRI	5	3.8	–	–	–	–
Oxaliplatin, irinotecan, 5-FU (any line)	30	23.1	–	–	–	–
Oxaliplatin, irinotecan, 5-FU, bevacizumab, cetuximab (any line)	16	12.3	12.8	31.7	4	3.0

3C6. For evaluation the same categorization was used as in the BR.21 study: samples with more than 1 percent of tumor cells showing membranous (partial or complete) staining of any intensity were stated as positive for EGFR.

RESULTS

Comparative analysis of clinical efficacy of different protocols

As first-line treatment, there was no difference in the clinical response in regard of time to progression between oxaliplatin and irinotecan based protocols (median TTP: 9.3 (n = 54) versus 10.7 months (n = 59) (p = 0.3); and OS: 32.7 versus 32.1 months *(Table 1, Figure 1).* Combinations with targeted biological therapies in unselected patient population achieved similar results between 10 and 10.2 months. There was a trend toward better survival if the chemotherapy was combined with biological therapy (bevacizumab or cetuximab): OS: 35.9 versus 36.7 months, but this difference

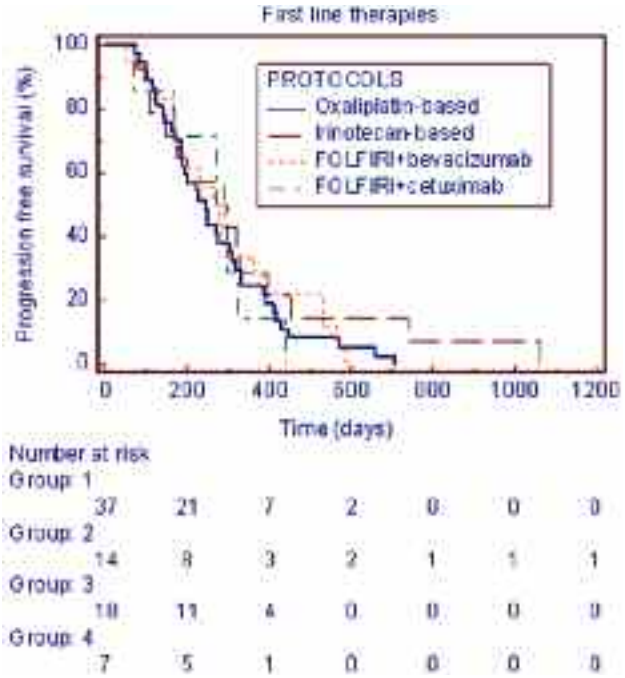

Figure 1. The four most frequent first line protocols (Kaplan-Meier analysis)

was not significant ($p > 0.5$), therefore we need biomarkers to identify patients who benefit most from biological therapies.

The median TTP of oxaliplatin- and irinotecan-based protocols were 8.1 (n = 24) and 6.6 months (n = 46) respectively, while protocols combining various chemotherapies with bevacizumab resulted in 5.8 months TTP (n = 19) and combining bevacizumab with FOLFIRI achieved also 5.8 months (n = 18). There was only 7 cetuximab chemotherapy combinations in this treatment line which is to low number to evaluate but these patients had longer, 10.1–12.4 months average TTP *(Table 1)*.

Thirty-seven patients out of the 130 received anti-EGFR antibody treatment (cetuximab or panitumumab) as monotherapy or in combination with chemotherapy *(Table 1).* The disease control rate (SD + PR + CR) of the anti-EGFR cetuximab monotherapy in the 2nd and later treatment lines was 25%.

In conclusion, in the unselected patient population it is difficult to make a decision which treatment protocol would be the most beneficial for an individual patient.

EGFR expression in colorectal cancer samples

The first biomarker criteria of anti-EGFR therapies was that at least 1% EGFR IHC positivity had to be detected with the standardized immunohistochemical assay or kit. The IHC status was available in 22 cases *(Table 2)*. Out of these 19 cases were positive (86%).

This is a very high rate considering that the disease control rate of cetuximab monotherapy was only 25% (see later). Therefore, our results indicate that EGFR IHC has a very weak predictive value. It is obvious that we need better biomarkers.

Frequency and type of KRAS mutations in Hungarian colorectal cancer patients

One hundred colorectal cancer samples were analyzed for KRAS mutations; 44% of these tumors contained mutations in exon 2 of the KRAS gene. Ninety-seven percent of these were one of the most frequent 7 mutations which are detected by the commercially available kits. This seems acceptable, however, even in this patient population we identified a patient with a mutation not among these 7 most frequent mutations. The high percentage of KRAS mutant patients forecasts KRAS testing as a much better biomarker.

Table 2. EGFR IHC results

Treatment with cetuximab (N = 37)	N (out of 37)	100% = 37	100% = 130
EGFR +	19	51.4	14.6
EGFR –	3	8.1	2.3
Unknown	15	40.5	11.5

Frequency and type of KRAS and BRAF mutations in patients treated with anti-EGFR therapy

Out of the 37 patients treated with anti-EGFR therapy *(Table 3),* samples from 30 patients were collected and out of these 26 contained sufficient amount of tumor cells for molecular analysis. However, in the case of 7 patients samples from metastasis or from relapsed tumor were available therefore overall we analyzed 38 samples of colorectal adenocarcinomas for KRAS exon 2 mutations. Out of these samples, in 30 samples of 25 patients BRAF exon 15 was analyzed as well. Activating mutations of KRAS were present in 42% of the patients (11/26) *(Figure 2)*.

Nine (82%) of them were codon 12 mutations and two of them were codon 13 mutations (G37C = G13R and G38A = G13D) *(Figure 3)*. The most frequent KRAS mutation was the transition of G35A = G12D (n = 6) that means the guanine (G) changes to adenine (A) at the 35th nucleotide in the genotype (in DNA) which exchanges the glycin (G) for an aspartate (D) in the phenotype (in protein). The other three codon 12 mutations were G35T = G12V (2 patients) and G34C = G12R transversions. The G35A, G35T and G38A are the most frequent KRAS mutations in colorectal adenocarcinoma.

Identification of a rare KRAS mutation

G37C is a rare but known mutation of this tumor type (ref: Sanger database). However, this mutation is not among the 7 most frequent mutations which are routinely tested in molecular pathology laboratories. The only in vitro diagnostic kit available, the DsX Therascreen does not detect this mutation. Therefore, clinical information with this mutation is very important. This patient received cetuximab monotherapy in the third line and experienced progressive disease during treatment. If we consider the unnecessary side effects and the extremely high cost of targeted therapies it may be reasonable to test for these relatively rare mutations as well.

Detection of a patient with BRAF mutation

Mutations in the BRAF gene occur in only 5% of colorectal cancers. However, KRAS and BRAF mutations are mutually exclusive therefore the frequency of BRAF mutations is 10% in KRAS wild-type patients. Our results are in line with these observations since we identified only one (4%) BRAF mutation in this patient pool *(Figure 2)*.

In case of 18 samples of 14 patients with wild-type KRAS tumors the frequency was 7.1%. The type of mutation was the T1799A = V600E transversion which is the most frequent (98%) BRAF mutation in colorectal adenocarcinomas (ref: Sanger database) *(Figure 4)*. This patient did not respond to cetuximab monotherapy and had short, 93 days TTP. This case reinforces the significance of BRAF testing in the negative selection of patients for anti-EGFR therapies.

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Table 3. Characteristics of patients receiving anti-EGFR treatments

Patients' characteristics with anti-EGFR cetuximab/panitumumab treatment	N (out of 37)	100% = 37		100% = 130	
Age					
< 65 years	17	45.9	100.0	13.1	28.5
≥ 65 years	20	54.1		15.4	
Gender					
Female	16	43.2	100.0	12.3	28.5
Male	21	56.8		16.2	
Stage					
I–III	21	56.8	100.0	16.2	28.5
IV	16	43.2		12.3	
Localization					
Colon	26	70.3	100.0	20.0	28.5
Rectum	11	29.7		8.5	
Histological grade					
G I–II	19	51.4	100.0	14.6	28.5
G III	6	16.2		4.6	
G X	12	32.4		9.2	
Metastasis localization					
Liver	25	67.6		19.2	
Lung	6	16.2		4.6	
Lymph nodes	9	24.3		6.9	
Peritoneum	1	2.7		0.8	
Other	6	16.2		4.6	
Treatment					
1st line only	3	8.1	100.0	2.3	28.5
1–2nd line	6	16.2		4.6	
3rd line or more	28	75.7		21.5	
Adjuvant treatment					
Yes	13	35.1	100.0	10.0	28.5
No	24	64.9		18.5	
Metastasectomy					
Yes	12	32.4	100.0	9.2	28.5
No	25	67.6		19.2	

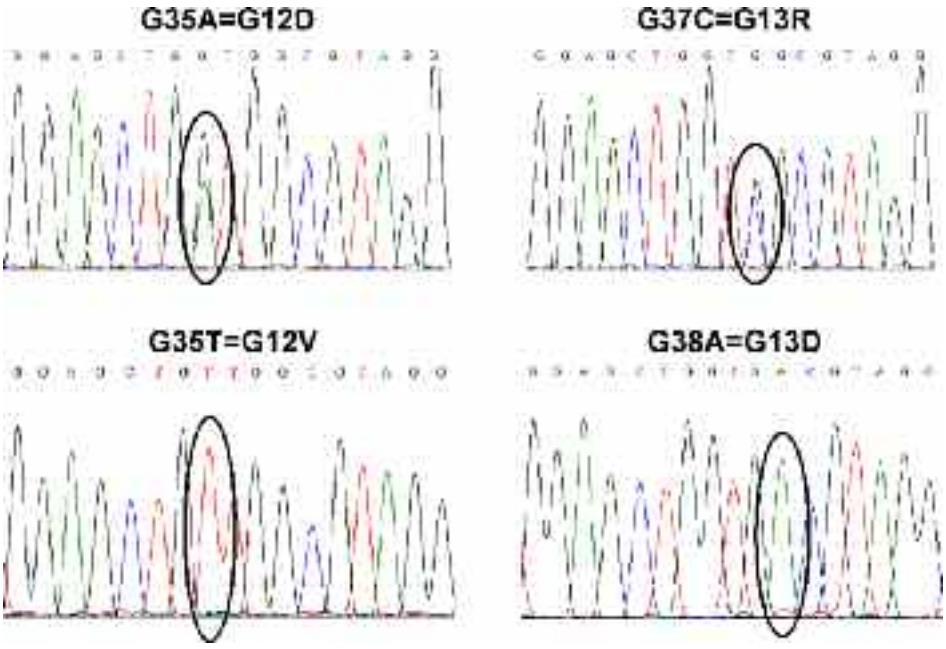


Figure 2. KRAS mutations (electrofluorogram of capillary sequencer)

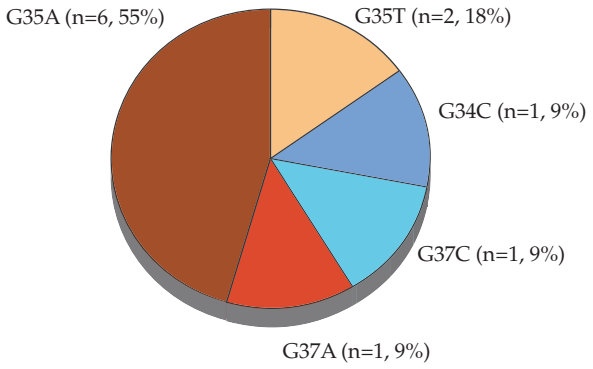


Figure 3. Frequency of different KRAS mutations

Analysis of the molecular status of KRAS and BRAF in multiple parallel samples

In case of 8 patients there were additional samples available for mutation status analysis besides the primary tumors *(Table 4).*

There were two liver metastases and two peritoneal metastases. In case of two patients one or more tumor relapses were also available and in one case liver metastasis and sample from the recurred tumor was also accessible. We could not detect any difference in the BRAF mutation status: every successful BRAF mutation analysis indicated wild-type status in this set of samples. We found difference in the KRAS status between different samples in case of one patient. Although the primary tumor contained a G35T = G12V mutation, we could not detect any KRAS mutation from the metastasis in peritoneum *(Figure 5).* This tumor did not give any response on cetuximab monotherapy. Another colorectal adenocarcinoma that caused a progressive disease despite the cetuximab monotherapy had a G35A = G12D mutation both in the primary tumor and in the three relapsed tumors *(Table 4, Figure 5).*

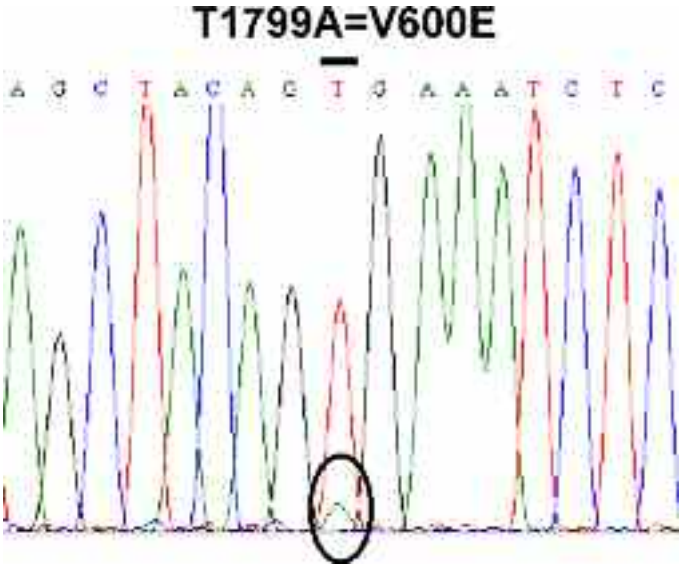
Anti-EGFR treatment of patients with KRAS/BRAF mutation status

Twenty-five patients with known KRAS mutation status received cetuximab therapy as monotherapy or in combination *(Table 5 and 6):* 11 got monotherapy: 1 in 2nd, 5 in 3rd line and 5 in 4th line. Thirteen patients were treated with cetuximab in combination with chemotherapy: 11 patients got FOLFIRI, one patient got FOLFOX and one patient got FOLFIRI and bevacizumab in addition to anti-EGFR therapy. One additional patient was treated with EGFR inhibitor in 3 lines: cetuximab + FOLFIRI in 1st line, cetuximab monotherapy in 3rd line and cetuximab + FOLFIRI + bevacizumab in 4th line. This patient had the BRAF mutation in his sample *(Table 5).*

There was a tendency of higher disease control rate and longer TTP in case of 1st and 2nd line treatment compared to the 3rd and 4th line therapy (CR + PR + SD ratio: 82% vs. 40%, average TTP: 337 vs. 184 days) *(Table 8).* The tendency was valid in the cetuximab monotherapy subgroup (CR + PR + SD ratio: 100% vs. 27%, average TTP: 328 vs. 144 days) and in the cetuximab + FOLFIRI subgroup (CR + PR + SD ratio: 78% vs. 67%, average TTP: 269 vs. 239 days), too. While cetuximab as a monotherapy was administered only once (8%) in 2nd line and in the other 11 patients in later treatment lines, in the case of cetuximab + FOLFIRI combination the ratio of the 1st + 2nd line treatment was 75% (9/12). Cetuximab + FOLFIRI treatment resulted in higher disease control rate and longer TTP than cetuximab alone (CR + PR + SD ratio: 75% vs. 33%, average TTP: 277 vs. 161 days) *(Table 6).*

Efficacy of cetuximab monotherapy in association with KRAS/BRAF mutation status

Since the patient with BRAF mutant tumor was treated with a 3rd line cetuximab monotherapy we had information about 12 patients with known KRAS/BRAF



‚Figure 4. V600E BRAF mutations (electrofluorogram of capillary sequencer)

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Table 4. KRAS status in multiple samples of the same patient

Patient	Id.	Anti-EGFR therapy		TTP (day)	Best response	BRAF status	Sample 1		Sample 2		Sample 3		Sample 4	
		Drug	Line				Sample type	KRAS status	Sample type	KRAS status	Sample type	KRAS status	Sample type	KRAS status
1	L05	cetuximab monoth.	4	?	PD	wild type	primary	wild type	liver met.	wild type				
2	L14	cetuximab monoth.	3	62	PD	wild type	primary	G12V	peritoneal met.	wild type				
3	L42	cetuximab monoth.	4	131	PD	wild type	primary	wild type	liver met.	wild type				
4	L45	cetuximab monoth.	3	192	PD	wild type	primary	G12D	relapse	G12D	relapse	G12D	relapse	G12D
5	L48	cetuximab monoth.	3	223	CR	wild type	primary	wild type	liver met.	wild type	relapse	wild type		
6	L96	cetuximab monoth.	4	87	PD	wild type	primary	wild type	lung met.	wild type				
7	L53	cetuximab + FOLFIRI	1	440	CR	wild type	primary	wild type	peritoneal met.	wild type				
8	L56		Ø			wild type	primary	wild type	relapse	wild type				

Table 5. Anti-EGFR therapies

Anti-EGFR treatment		Total	1st line	2nd line	3rd line	4th line	Best response: CR	Best response: PR	Best response: SD	Best response: PD	KRAS M+	BRAF M+	KRAS/BRAF M–
Cetuximab	N	11		1	5	5	1	0	2	8	4		7
	Av. TTP (day)	166.9		328	131.8	170.5	223		358	104.3	104.25		208.7
Cetuximab + FOLFIRI	N	11	6	2	1	2	2	1	5	3	6		5
	Av. TTP (day)	273.2	256.2	366.5	374	172	363	323	273.6	157.5	283.8		262.6
Cetuximab + FOLFOX	N	1			1				1		1		
	Av. TTP (day)	420			420				420		420		
Cetuximab + FOLFIRI / Cetuximab / Cetuximab + FOLFOX + bevacizumab	N	1	1		1	1		1		1		1	
	Av. TTP (day)	148.7	296		97	53		296		97		148.7	
Cetuximab + FOLFIRI + bevacizumab	N	1	1					1					1
	Av. TTP (day)	728	728					728					728
Total	N	25	8	3	8	8	3	3	8	12	11	1	13
	Av. TTP (day)	248	329.3	353.7	193.8	154.1	316	449	278	127	225.6	148.7	274.4

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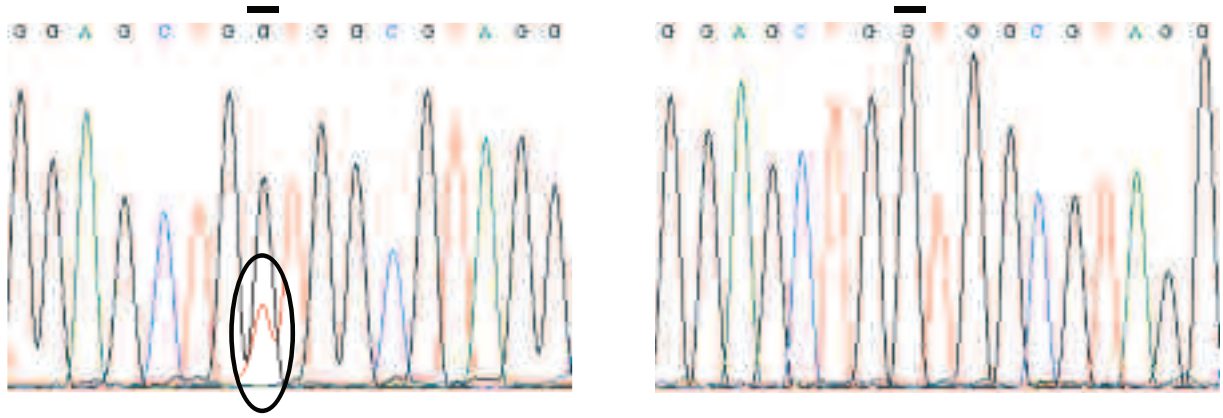

Figure 5. KRAS mutation is present in primary but absent in the metastasis of the same patient

Table 6. Anti-EGFR treatments by lines of treatment

Line of anti-EGFR treatment		Total	All treatments				Cetuximab monotherapy				Cetuximab + FOLFIRI			
			CR	PR	SD	PD	CR	PR	SD	PD	CR	PR	SD	PD
1st	N	8	2	3	1	2					2	2	1	2
	Av. TTP (day)	329.3	363	449	166	66					363	310	166	66
2nd	N	3			3				1				2	
	Av. TTP (day)	353.7			353.7				328				366.5	
3rd	N	8	1		3	4	1			5			1	
	Av. TTP (day)	193.8	223		297	109	223			86.8			374	
4th	N	7			2	5			1	4			1	1
	Av. TTP (day)	171			242	136			388	98			95	249
Total	N	26	3	3	9	11	1		2	9	2	2	5	3
	Av. TTP (day)	248	316	449	289	116	223		239	91.6	363	310	274	158

mutation status treated with cetuximab monotherapy *(Table 7)*. The therapy was more effective in KRAS/BRAF wild-type patients: there were one complete response (CR, RR = 14%), two SDs (disease control rate: 42.8%) and 4 progressive diseases (PD, 57%) in this subgroup. The average TTP was 208.7 days. There was no response in the patients with KRAS mutant tumors: all of them (5/5 = 100%) had PD with an average TTP of only 104 days.

There was a patient with PD whose first CT control was missed. If we omit this patient from the dataset, the difference between the TTP in KRAS/BRAF WT and KRAS M + /BRAF M + patients is significant (p = 0.02) *(Figure 6)*.

Table 7. Results of cetuximab monotherapy

Mutation status		Total	1st	2nd	3rd	4th	Best response % (N)			
			line				CR	PR	SD	PD
KRAS Mut +	N %	4			3	1				100% (4)
	Av. TTP (day)	104.3			113.7	76				104.3
BRAF Mut +	N %	1			1					100% (1)
	Av. TTP (day)	97			97					97
KRAS/BRAF Mut–	N %	7		1	2	4	14% (1)		29% (2)	57% (4)
	Av. TTP (day)	208.7		328	159	202	223		358	104.3
Total	N %	12		1	6	5	8% (1)		25% (3)	67% (8)
	Av. TTP (day)	160.5		328	126	170.5	223		239	118.1

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Table 8. Results of FOLFIRI + cetuximab therapy

Mutation status		Total	1st	2nd	3rd	4th	Best response % (N)			
			line				CR	RP	SD	PD
KRAS Mut+	N %	6	3	1	1	1	17% (1)	17% (1)	50% (3)	17% (1)
	Av. TTP (day)	283.8	304.5	341	374	95	286	323	270	?
BRAF Mut+	N %	1	1					100% (1)		
	Av. TTP (day)	296	296					296		
KRAS/BRAF Mut-	N %	5	3	1		1	20% (1)		40% (2)	40% (2)
	Av. TTP (day)	262.6	224	392		249	440		279	157.5
Total	N %	12	7	2	1	2	17% (2)	17% (2)	42% (5)	25% (3)
	Av. TTP (day)	276.5	262.8	367	374	172	363	309.5	275	157.5

Efficacy of cetuximab + FOLFIRI in association with KRAS/BRAF mutation status

Since the patient with BRAF mutant tumor was treated with 1st line cetuximab + FOLFIRI we had information about 12 patients with known KRAS/BRAF mutation status treated with this drug combination *(Table 3)*. The KRAS/BRAF wild-type tumor status was not associated with accelerated therapeutic efficacy: there were one CR (RR = 20%), 2 SDs (disease control rate: 60%) and 2 PDs (40%) in this subgroup. The average TTP was 263 days. In patients with KRAS mutant tumors there were one CR, one PR (RR = 33%), 3 SDs (disease control rate: 83%) and only one PD (17%). The average TTP was 284 days in this subgroup. In case of the patient with BRAF mutant tumor the cetuximab and FOLFIRI administration resulted in SD with a TTP of 296 days *(Table 8)*.

Frequency of PIK3CA mutations by high resolution melting analysis

Thirty-six samples of 25 colorectal cancer patients were analyzed for mutations in exon 9 and exon 20 of PIK3CA. PIK3CA mutations showed very high heterogeneity in multiple parallel samples. In two cases the primary tumor had exon 20 mutation but the liver metastasis was wild type. In one case the primary tumor had mutations in both exons but the lung metastasis was wild type. In two cases the primary tumor was wild type but the relapsed tumor had mutations. There was one case in which both the primary tumor and the metastasis were wild type. Thirteen patients had only wild type and 11 (46%) patients had mutations in either or both exons in any samples. If we count primary tumors only, there were 9 PIK3CA mutant patients (37.5%) (3 exon 9, 3 both exons, 3 exon 20 mutations). The overall frequency of PIK3CA mutations was 12 out 36 samples (33%). PIK3CA and KRAS mutations were independent. There were 4 PIK3CA mutants out of 10 KRAS mutants (40%) and 6 out of 14 KRAS wild-type (43%) of primary tumors. Twenty-nine percent of tumors were wild type for all the three markers (KRAS, BRAF and PIK3CA). This could very well correspond to the group of patients who benefit from cetuximab monotherapy (25%). However, it is difficult to evaluate the clinical value of PIK3CA due to the heterogeneity of tumors for this oncogene. Notably, in the case who responded with complete remission to cetuximab monotherapy, there was an exon 20 PIK3CA mutation in the primary tumor, but the metastasis was wild-type. This observation may indicate that, in contrast to KRAS, the primary tumor is not suitable for the analysis of PIK3CA mutations if we wish to predict the response of the distant metastasis.

LIST OF MAJOR FINDINGS

- The different first-line (oxaliplatin- and irinotecan-based) chemotherapy protocols achieve the same clinical response in metastatic colorectal cancer patients: TTP: 9.3 versus 10.7 months (p = 0.3); and OS: 32.7 versus 32.1 months.
- There is trend toward better survival if the chemotherapy is combined with biological therapy (bevacizumab or cetuximab): OS: 35.9 versus 36.7 months. However, this difference is not significant (p > 0.5) therefore we need biomarkers to identify patients who benefit most from biological therapies.

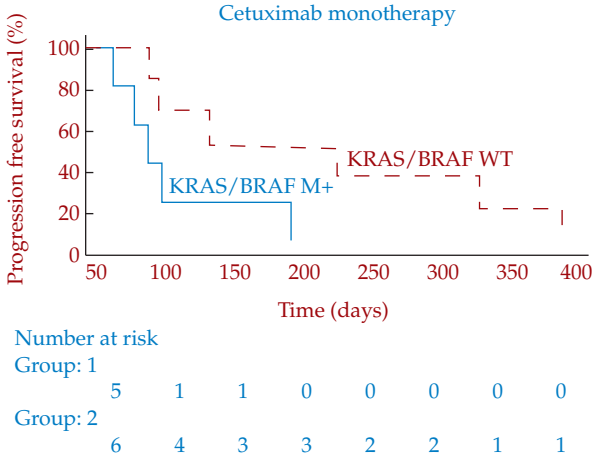

Figure 6. Results of cetuximab monotherapy (Kaplan-Meier curve)

- The disease control rate (SD + PR + CR) of the anti-EGFR cetuximab monotherapy in the 2nd and later treatment lines was 25%.
- EGFR expression was detected by immunohistochemistry in 86% of patients. Therefore, EGFR IHC is not suitable to select patients for individualized anti-EGFR therapy.
- The frequency of KRAS mutations in our patient population was 44.3% (n = 100).
- There was no significant difference in the clinical response between KRAS mutant and wild-type tumors to chemotherapy and cetuximab combination therapy. However, none of the patients with KRAS mutant tumor responded to cetuximab monotherapy. The disease control rate in the KRAS wild-type patients was 43% and the TTP was twice longer (208 days) than in the KRAS mutants (104 days). Therefore, KRAS is a useful but not sufficient biomarker to select patients for anti-EGFR monotherapy.
- The primary tumor is suitable for KRAS analysis because there was only 1 out 7 cases in which the KRAS status was different in the primary and the metastatic tumor in the same patient.
- We have identified a patient who had a rare type of KRAS mutation (G37C). This patient did not respond to anti-EGFR therapy. Clinical database of rare mutation is warranted to collect information on the predictive value of these rare mutations.
- One BRAF mutant (V600E) patient was identified among the 36 patients treated with cetuximab (4%). This patient did not respond to anti-EGFR therapy.
- Thirty-six percent of colorectal tumors had mutations in exon 9 and exon 20 of PIK3CA analyzed by high resolution melting analysis. PIK3CA mutations occurred independently of the KRAS status.
- In 5 out of 7 cases the PIK3CA mutation status was different in the primary tumor and the metastasis therefore the primary tumor is not suitable for the prediction of the PIK3CA mutation status.
- Twenty-nine percent of colorectal tumors are wild type for all the three biomarkers (KRAS + BRAF + + PIK3CA). If the clinical value of these is further clarified, the combination of these biomarkers is potentially sufficient to identify patients who benefit most from anti-EGFR therapy.

DISCUSSION

As a result of molecularly targeted anti-EGFR therapies (cetuximab and panitumumab) complementing chemotherapy, liver metastasis can reduce in size and become operable in certain patients, which can contribute to the complete recovery of the patient. The main problem, however, is the fact that a positive response only occurs in one third of the patients, even in the case of chemotherapy combined protocol, and the side effects are considerable. As currently there is no precise predictive molecular diagnostics at our disposal, oncologists have to make one of two choices: they treat a large number of patients with anti-EGFR which has negative effects on the quality of life and also reduces the patient's chances of getting appropriate treatment or, if the oncologists refuse to take risks, they omit the use of anti-EGFR treatment in which case those patients for whom this would have been the appropriate treatment are also denied the chance of short-term survival or recovery.

The signal transduction pathways of epidermal growth factor receptor are subject to intensive research in the case of colon cancer as well. EGFR is expressed in the case of 30–85% of colon cancer patients. Data relating to gene amplification are controversial; in most of the cases the polysomy of chromosome 7 is accountable for multiple gene copies. The loss of one of the gene copies can also occur, EGFR-LOH (8%), which does not result in the protein deficiency of EGFR. In the case of colon cancer the best-known anti-EGFR strategies are monoclonal antibodies produced against the extracellular domain and the small molecule tyrosine kinase inhibitors (TKI).

Cetuximab (Erbitux®) is one of the monoclonal antibodies, which was licensed by the FDA in 2004 in the case of EGFR-positive metastatic colon cancer patients in monotherapy, irinotecan-resistant patients, or in combination with irinotecan for tumors that have developed resistance to irinotecan. Once cetuximab binds to the receptor, the latter becomes internalized and disintegrates and, as a result, it does not phosphorylate nor does it activate. This results in a considerable drop of EGFR concentration on the cell surface and thus the likelihood of signal transduction pathways working also diminishes. As demonstrated by experiments, cetuximab combined with irinotecan is more successful in irinotecan-resistant patients than a purely cetuximab-based treatment. At its first application a combination of oxaliplatin-based (FOLFOX, ACROBAT-test) and irinotecan (FOLFIRI) based treatment was examined.

Patients only if their tumor sample was positive in at least 1% for EGFR IHC were included into clinical trials on which the registration of cetuximab was based. Therefore, this test became obligatory condition of cetuximab treatment in colon cancer. But later clinical tests point out that there is no link between efficacy and the degree of immunohistochemical positivity. According to the Salt's working group there was also a 25% therapeutic response rate among immunohistochemically negative patients. The prestigious NCCN (National Comprehensive Cancer Network) now does not recommend EGFR IHC testing: „EGFR testing has not demonstrated predictive value, and therefore routine EGFR testing is not recommended. No patient should be included or excluded from cetuximab or panitumumab therapy on the basis of EGFR test result." In Hungary, EGFR IHC testing is still mandatory, but we

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consider the tumor EGFR positive even if only a single cell is IHC positive. The contradiction in immunohistochemical findings is so diverse that many times the presence of EGFR is not required for the selection of patients in therapeutic efficacy in clinical assessments. A reason behind this fact could be that the EFGR expression of primary tumors and metastasis can differ although many contradict this. The other possible explanation holds that immunohistochemical reaction is not sensitive enough or that the method is not fine-tuned for the needs of the samples. The problem could also be connected to the production of diagnostically used antibodies. The antibody is produced with A431 cell line with unusually high EGFR expression. It has been demonstrated in the cell line that in 95% it expresses low-affinity and in 5% high-affinity EGFR. It has been suggested that the biological effect is to be linked to the high-affinity receptor. The test makes no difference between the two groups and as a result it could be possible that the tumor only contains the receptor below measurement levels, but which is of high-affinity and as such responds to treatment. It could also be possible that a tumor expresses EGFR at detectable levels but that this level is not the primary signal path of survival and thus treatment remains inefficient.

The EGFR status, defined by various methods (FISH, real-time RT-PCR, ELISA and IHC), were compared because of the immunohistochemical findings. As a result of the test it has been revealed that there is little coincidence between EGFR statuses defined by measuring DNA, RNA or protein levels.

The identification of readily usable molecular markers predicting cetuximab sensitivity is of utmost importance for enhancing therapeutic response. When examining the components of signal paths originating from EGFR the cell lines containing PIK3CA activating mutations or PTEN deficient were revealed to be resistant to cetuximab in contrast with those cell lines where PIK3CA is wild-type or anti-PTEN functions are present.

The PI3K activity is enhanced by the somatic mutation of PI3K catalytic subunit: PIK3CA, and thus the signal paths are activated independently of EGFR, and the same process occurs when PTEN function is missing and also in the case of KRAS and BRAF mutant tumors. Upon sporadic examination of CRC patients the various working groups revealed 13.6–31.6% PIK3CA mutation. The ratio of PTEN mutation was found to be 41% by the Frattini working group in the case of cetuximab treated mCRCs, the prevalence of KRAS mutation is 30–40%, BRAF mutation is rarer: 0–12.5%, KRAS and BRAF mutations are mutually exclusive genetic events and their co-occurrence in tumors was registered at 0–0.4%. Lievre's working group examined 30 cetuximab-treated patients. None of the 11 treated responders had KRAS mutation, while 68.4% of the 19 resistant tumors were mutant ($p = 0.0003$). KRAS wild-type entailed long-term survival ($p = 0.016$; 16.3 versus 6.9 months). Cetuximab was administered with FOLFIRI in 3 cases, with irinotecan in 7 cases and was administered as monotherapy in one case. Based on several tests it can be stated that the activating KRAS mutation is an absolute negative predictive marker in the case of anti-EGFR antibody therapy for CRC patients. When further examining the above mentioned signal components it was observed that those cell lines which are PIK3CA mutant or PTEN deficient and are KRAS or BRAF mutants show a stronger resistance to cetuximab than those that are not dual mutants or PTEN deficient. It can be deduced that the constitutive and simultaneous activation of the KRAS and PIK3CA signal path triggers maximal resistance to the medication. The PTEN expression test of the tumor and the identification of PIK3CA, KRAS and BRAF mutations help in forecasting the efficacy of the treatment. The link between the mRNA level of proteins in the EGFR signal pathway and the effect of cetuximab was investigated in mCRC patients. The lower mRNA level of proteins in the case of COX-2, EGFR and IL-8 entails longer survival (13.5 vs. 2.3 months, $p = 0.028$). The high VEGF gene expression level co-occurs with cetuximab resistance. According to gene expression tests, those mCRC patients with tumors that have high expression level of EGFR ligand amphiregulin and epiregulin respond better to cetuximab treatment than those with lower expression levels.

Panitumumab (Vectibix®), the other well-known anti-EGFR antibody, which is a complete monoclonal human antibody which recognizes the ligand binding section, inhibits ligand attachment and stimulates receptor internalization but hinders its degradation and, as a result, the receptor can return to the cell surface again. As it is absolutely human antibody it does not induce anti-mouse immune response as opposed to cetuximab. Administered in monotherapy to therapy-resistant, metastatic EGFR-positive colon cancer patients it prolonged TTP by 50%. Currently it is used as second-line monotherapy in combination with chemotherapy products in case of EGFR-positive, metastatic CRC. The use of panitumumab has proved to be favorable for colon cancer patients regardless of age, gender, EGFR status and the type of previous chemotherapies. KRAS mutation induces resistance in the case of panitumumab treatment, similarly to cetuximab.

The development and practical use of molecular diagnostic methods hopefully will gain more and more importance in future for the selection of the most eligible patients for EGFR inhibitor targeted treatment.

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PUBLICATIONS

List of own publications related to the theses

1. Landherr L, Nagykálnai T, Mészáros E. A colon carcinoma adjuváns kezelése. Uzsoki utcai levelek 7: 31–35, 2001
2. Varga Sz, Póti Zs, Landherr L. Előrehaladott colorectalis carcinoma kemoterápiája. Uzsoki utcai levelek 7: 37–42, 2001
3. Landherr L. A vastagbéldaganatok korszerű kezelése. Orvostovábbképző Szemle Különszám: 17–19, 2004
4. Landherr L. Mészáros E. Végbél és anális csatorna rákok radio-kemoterápiája. Uzsoki utcai levelek 10: 37–44, 2005
5. Landherr L. Klinkó T. Patonay P. Colorectalis daganatok perorális kemoterápiájának racionalitása. Uzsoki utcai levelek 11: 45–50, 2007
6. Landherr L. Metastatic colorectal carcinoma (mCRC) specific therapy, VEGF inhibition. Orvosi és Gyógyszerészeti Szemle Targu Mures 54:181–184, 2008
7. Landherr L. Predictive biomarkers in the treatment of colorectal cancers. Orvosi és Gyógyszerészeti Szemle Targu Mures 55: 125–128, 2009
8. Landherr L. Nagykálnai T. Időskorú kolorektális daganatos betegek gyógyszeres kezelése. Magyar Onkológia 53: 97–105, 2009
9. Landherr L. Nagykálnai T. A távoli áttétes kolorektális rák (MCRC) első választású kezelésének fejlődése. Magyar Onkológia 53: 237–246, 2009
10. Landherr L. A gyomordaganatok nem sebészi kezelése. Primer májdaganatok és colorectális tumorok okozta májáttétek nem sebészi kezelése. GASTRO UPDATE 2009. Gastro Update Alapítvány, 2009, pp. 127–140, pp. 298–313
11. Landherr L. Áttétes colorectalis carcinoma (mCRC) célzott biológiai kezelése, az EGF gátlás. Orvostudományi értesítő, Targu Mures 82:228–231, 2009

Book chapters

1. Landherr L. Gastrointestinális tumorok. Gastro Update Grossarl. Melánia Kiadó Budapest, 2000, pp. 276–287
2. Landherr L. Onkológia. Gastro Update 2001. Melánia Kiadó, Budapest, 2001, pp. 61–64, pp. 90–96, pp. 206–210, pp. 281–283
3. Landherr L. A gyomordaganatok nem sebészi kezelése. Primer májdaganatok és colorectális tumorok okozta májáttétek nem sebészi kezelése. GASTRO UPDATE 2009. Gastro Update Alapítvány. 2009, pp. 127–140, pp. 298–313

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