KRAS mutations in Slovene patients with colorectal cancer: frequency, distribution and correlation with the response to treatment

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Abstract. *KRAS* mutations are proved as a predictor of response to EGFR-targeted therapies for patients with metastatic colorectal cancer. For identifying the wild-type *KRAS* (wt-*KRAS*) responder subset of patients who will benefit from novel agents our laboratory has introduced the TheraSreen K-RAS Mutation Kit® an allele-specific RT-PCR based assay. Our aim is to describe the validation procedure of this method in our laboratory, determine the portion of colorectal cancer patients with wt-*KRAS* status, and assess the prognostic power of mutational status for the anti-EGFR therapy outcome in colorectal cancer patients. In this study 302 samples from 273 patients with metastatic colorectal cancer were tested for 7 most common mutations on codon 12 and 13 of the *KRAS* gene. We used HT-29 and CCL-247 cell lines to determine the sensitivity of the method for different proportions of tumor cells in the sample. We determined that 2% of cells carrying a *KRAS* mutation must be present in the sample for an undisputable detection of mutated signal using the LightCycler Adapt Software. Among the tested patients 54.5% had a wt-*KRAS* genotype and 45.5% had a mutated *KRAS* genotype. The p.Gly12Asp was the most common detected mutation (38.5%). Among the cetuximab therapy responders, 85.7% had a wt-*KRAS* genotype. We have shown that the RT-PCR method introduced to discriminate between anti-EGFR therapy responders and non-responders is efficient, reliable and quickly applicable. The ratio of mutated versus wt-*KRAS* patients in our study is similar to ratios reported by other authors, as is the high correlation between wt-*KRAS* genotype and response to cetuximab therapy. Nevertheless the selection of patients for treatment solely on the basis of KRAS status is not perfect due to the fact that some responders are among the patients with mutated KRAS and some non-responders among the wt-KRAS patients.

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Inroduction

The epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase receptor. It is expressed in epithelial tissues and acts as a cell growth promoter. The EGFR activates at least three major signaling pathways: the RAS-MAPK, the P13K-Akt and the STAT pathway (1). According to the literature the EGFR contributes to the development and progression of several types of cancer, cancer of the head and neck, ovarian, cervical, bladder, esophageal, gastric, breast, endometrial, lung and colorectal cancer (1). EGFR is overexpressed in 50-80% of colorectal tumors (2-6) and is therefore a suitable target for anti-cancer therapies. Currently two strategies to attenuate EGFR signaling are in use: monoclonal antibodies that bind to the ligand-binding domain and inhibit the binding of specific ligand (cetuximab and panitumumab) (7) or small EGFR tyrosine kinase inhibitor molecules that bind to the intracellular domain of EGFR and by competing for binding with ATP inhibiting tyrosine phosphorylation (gefitinib, erlotinib) (8). Nevertheless, the anti-EGFR therapies are only effective in a subset of patients with colorectal cancer (9). To optimize benefits and reduce the risk of anti-EGFR therapies, the EGFR as well as the molecules involved in its signaling pathway have been evaluated as potential markers for predicting therapy outcomes. Recent studies demonstrated that *KRAS* mutations proved as a predictor of response to EGFR-targeted therapies for patients with metastatic colorectal cancer (10-14).

The *KRAS* gene encodes the human cellular homolog of a transforming gene isolated from the Kirsten rat sarcoma virus. The product of the gene is a small G-protein that functions downstream of EGFR-induced cell signaling. It belongs to the family of RAS proteins that are GDP/GTP-binding proteins that act as intracellular signal transducers by coupling the signal from the cell surface receptors to intracellular targets (15,16). The human *KRAS* proto-oncogene is mutated in 30-40% of colorectal cancers. The most frequent alterations are detected in codons 12 and 13 in exon 2 of the *KRAS* gene (17-19). The *KRAS* mutations represent an early event in the development and progression of colorectal cancer (20-22). The protein product of mutated KRAS gene has an increased binding affinity for GTP, causing accumulation in the active GTP-bound state by impairing intrinsic GTPase

activity and conferring resistance to GTPase-activating proteins (23). The signal transduction therefore no longer runs via EGFR, resulting in the fact that patients with *KRAS* mutations on codon 12 and 13 have poor response to therapy with anti-EGFR inhibitors.

For identifying the wt-*KRAS* responder subset of patients who will benefit from novel agents targeting the EGFR, a suitable tool for detecting *KRAS* mutations is required in a laboratory. Our laboratory has introduced a CE-marked TheraScreen K-RAS mutation kit (Roche Applied Science, Mannheim, Germany); an allele-specific RT-PCR based assay. This kit has been approved as *in vitro* diagnostic device by the EMEA (European Medicines Agency) (24).

Therefore, the objectives of this article are 1) to describe the validation procedure of the TheraScreen method in our laboratory, 2) to determine the portion of colorectal cancer patients with wt-*KRAS* status, and 3) to assess the power of mutational status for prognostication of the anti-EGFR therapy outcomes in colorectal cancer patients.

Materials and methods

Patients and tumor samples. In this study 302 samples from 273 patients with metastatic colorectal cancer, treated at the Institute of Oncology Ljubljana from August 2008 to September 2009, were tested for diagnostic purpose. Apart from tumor samples, in some cases, the metastatic tissue samples from the patients were also obtained, with written consent, and tested.

The appropriate formalin-fixed paraffin-embedded tumor tissue block was selected by the appointed pathologist, who also evaluated the percentage of tumor cells in the paraffin slides from the first and last HE-stained cut.

For the validation of the TheraScreen method and assessment of power of mutational status for prognostication of the anti-EGFR therapy outcomes we collected samples from 23 patients. For this reason 30 samples were tested altogether: 25 tumor samples (additional tumor sample was available for two patients) and 5 pair matched non-tumor samples. These 23 patients with metastatic colorectal cancer were treated with cetuximab and chemotherapy as the second, third- or further-line treatment after failure of the treatment with irinotecan. Of the 23 patients treated with cetuximab, 11 were male and 12 were female with the age ranging from 33 to 72 years. The patients were regularly followed up once a week for at least three months after the beginning of the therapy with cetuximab. The response to treatment was evaluated according to the RECIST criteria.

Cell lines. The HT-29 are colorectal adenocarcinoma cells that do not harbor mutations in the KRAS gene. They were grown in advanced EMEM supplemented with 10% FCS at 37˚C and 5% CO_2. The CCL247 are colorectal carcinoma cells that harbor the p.Gly13Asp mutation of the KRAS gene and were used as a positive control in mutation assays. They were grown in McCoy's 5A-modified medium at 37˚C and 5% CO_2.

The mononuclear cells were isolated from fresh peripheral blood with Ficoll-Paque Premium (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The fresh blood sample was diluted with 1X PBS and layered on the Ficoll-Paque Premium. The tubes were centrifuged at 4000 rpm for 30 min. The layer of mononuclear cells was collected and washed with PBS three times. Series of different cell mixture samples was prepared to mimic the heterogeneous tumor tissue from a patient. The DNA from the cell mixtures was then isolated and RT-PCR with TheraScreen K-RAS Mutation Kit was performed.

DNA extraction. Non-colored paraffin-embedded sample tissue cuts with the thickness of 10 μm (approximately 100 μm of tumor tissue) were prepared in an eppendorf tube by the pathologist. The tissue was de-paraffinised by washing with Xylol and rehydrated by washing with 100% ethanol. The tissue was dried to evaporate all ethanol. For the DNA extraction High Pure PCR template preparation kit (Roche) was used according to the manufacturer's protocol. After the isolation DNA concentration was spectrophotometrically measured at 280/260 nm.

KRAS mutation analysis. For determining the 7 most common mutations on codon 12 and 13 of the *KRAS* gene we used the TheraSreen K-RAS Mutation Kit (Roche Applied Science). The RT-PCR was performed according to the manufacturer's instructions (25) with a modification in the amount of the sample DNA added. For the testing 100 ng of DNA was added per reaction well. The assay was run on the Roche LightCycler 480 real-time PCR System Instrument II (Roche Applied Science). The results were analyzed using the Light Cycler® Adapt Software (Roche Applied Science).

Statistical analysis. The Statistic Online Computational Resource (SOCR) tool was used for statistical analysis. The Pearson's Chi-square test was used to calculate the p-value for association between *KRAS* mutation and response to cetuximab. The statistical significance was set at $p<0.05$.

Results

Validation procedure for the TheraScreen diagnostic method. For establishing the *KRAS* genotype testing in our laboratory we tested a panel of 30 retrospective samples from 23 patients (5 of the tested tumor samples had matching non-tumor samples) diagnosed with metastatic colorectal carcinoma with the TheraSreen K-RAS Mutation Kit. We compared the results of TheraSreen K-RAS Mutation Kit method with the results of pyrosequencing. The pyrosequencing was performed by the appointed reference laboratory at the Institute of Pathology of the LMU Munich.

DNA isolated from the peripheral blood of a healthy individual was used as a negative control and the DNA isolated from the CCL247 colorectal carcinoma cell line carrying the *KRAS* p.Gly13Asp mutation as a positive control. The DNA from all the samples was successfully extracted and was of suitable quality for PCR amplification. We detected no mutations in the non-tumor samples from the patients. Of the 25 tested tumor samples 12 (48%) were positive for *KRAS* mutations. The positive control sample (CCL247 cell line) tested positive for *KRAS* p.Gly13Asp mutation, while the negative control sample (blood sample from a healthy individual) gave a wt-*KRAS* result.

Table I. Results for *KRAS* genotyping tested with TheraSreen K-RAS Mutation Kit compared with the results of pyrosequencing.

No.	Sample ID	Sample type	TheraScreen Real-time PCR	Pyrosequencing Amplicon sequencing
1	KR26	Non-tumor	wt	wt
2	KR27	Non-tumor	wt	wt
3	KR28	Non-tumor	wt	wt
4	KR29	Non-tumor	wt	wt
5	KR30	Non-tumor	wt	wt
6	KR31	Whole blood	wt	
7	KR1	Tumor	wt	wt
8	KR2	Tumor	wt	wt
9	KR5	Tumor	wt	wt
10	KR9	Tumor	wt	wt
11	KR10	Tumor	wt	wt
12	KR12	Tumor	wt	wt
13	KR15	Tumor	wt	wt
14	KR16	Tumor	wt	wt
15	KR17	Tumor	wt	wt
16	KR18	Tumor	wt	wt
17	KR21	Tumor	wt	wt
18	KR22	Tumor	wt	wt
19	KR23	Tumor	wt	wt
20	KR20	Tumor	12ARG	12ARG
21	KR3	Tumor	12ASP	12ASP
22	KR6	Tumor	12ASP	12ASP
23	KR13	Tumor	12ASP	12ASP
24	KR14	Tumor	12ASP	12ASP
25	KR25	Tumor	12ASP	12ASP
26	KR4	Tumor	12SER	12SER
27	KR7	Tumor	12SER	12SER
28	KR8	Tumor	12VAL	12VAL
29	KR11	Tumor	13ASP	13ASP
30	KR19	Tumor	13ASP	13ASP
31	KR24	Tumor	13ASP	13ASP
32	KR32	Cell line CCL247	13ASP	

In our laboratory the determined sensitivity and specificity of the RT-PCR with the TheraScreen K-RAS Mutation Kit method is 100%, as we detected no false positive or false negative samples. This was confirmed by pyrosequencing since the data from real-time PCR and pyrosequencing testing is in 100% concordance for the tested samples (Table I).

According to the manufacturer, TheraSreen K-RAS Mutation Kit is able to detect 1% of mutated DNA in a background of wt-DNA. With the intention to confirm the given manufacturer's detection limit (and corresponding sensitivity), the method was tested in our laboratory. To set up the minimum percentage of tumor cells carrying the KRAS p.Gly13Asp mutation in a background of non-tumor cells that are required for successful mutation detection, a series of cell mixture samples was prepared to mimic the heterogeneous tumor tissue from a patient (Table II). We determined that at least 2% of CCL247 cells carrying the p.Gly13Asp mutation must be present in the sample mixture for successful positive detection by the LightCycler Adapt Software. Nevertheless, when we performed the analysis manually, the apparent amplification of mutated DNA started when 0.2% of CCL247 carrying KRAS p.Gly13Asp mutation was present in the sample mixture. We observed steeper amplification curves as the percentage of tumor cells in the sample increased (Fig. 1).

We also prepared mixtures of DNA isolated from HT-29 (without *KRAS* mutation) and CCL247 (harboring the p.Gly13Asp mutation in the *KRAS* gene). In this case also at least 2% of CCL247 DNA carrying the p.Gly13Asp mutation must be present in the sample for successful positive detection by the LightCycler Adapt Software.

Table II. Sensitivity of test, determined in samples containing different proportions of mutated cells.

No. of HT-29 wt KRAS cells	No. of CCL247 cells harboring the KRAS p.Gly13Asp mutation	No. of mononuclear cells	KRAS genotype
$1x10^6$ (4.95%)	$1x10^4$ (0.05%)	$1.9x10^7$ (95%)	wt
$2x10^6$ (9.9%)	$2x10^4$ (0.1%)	$1.8x10^7$ (90%)	wt
$4x10^6$ (19.8%)	$4x10^4$ (0.2%)	$1.6x10^7$ (80%)	wt
$8x10^6$ (39.6%)	$8x10^4$ (0.4%)	$1.2x10^7$ (60%)	wt
$10x10^6$ (49.5%)	$10x10^4$ (0.5%)	$1x10^7$ (50%)	wt
$12x10^6$ (59.4%)	$12x10^4$ (0.6%)	$0.8x10^7$ (40%)	wt
$14x10^6$ (69.7%)	$14x10^4$ (0.7%)	$0.6x10^7$ (30%)	wt
$16x10^6$ (79.2%)	$16x10^4$ (0.8%)	$0.4x10^7$ (20%)	wt
$18x10^6$ (89.1%)	$18x10^4$ (0.9%)	$0.2x10^7$ (10%)	wt
$20x10^6$ (99%)	$20x10^4$ (1%)	/	wt
$1.96x10^6$ (98%)	**$4x10^4$ (2%)**	**/**	**p.Gly13Asp**
$1.94x10^6$ (97%)	$6x10^4$ (3%)	/	p.Gly13Asp
$1.9x10^6$ (95%)	$10x10^4$ (5%)	/	p.Gly13Asp
$1.8x10^6$ (90%)	$20x10^4$ (10%)	/	p.Gly13Asp
$1.6x10^6$ (80%)	$40x10^4$ (20%)	/	p.Gly13Asp
$1.4x10^6$ (70%)	$60x10^4$ (30%)	/	p.Gly13Asp

HT-29 colorectal adenocarcinoma cell line with no *KRAS* mutation, CCL247 colorectal carcinoma cell line carrying KRAS p.Gly13Asp mutation and mononuclear cells isolated from the peripheral blood of a healthy individual were mixed in appropriate volumes. The range of mutated cells was 0.05-30%. The total amount of cells in a mixed sample was $2x10^7$ cells or $2x10^6$ cells.

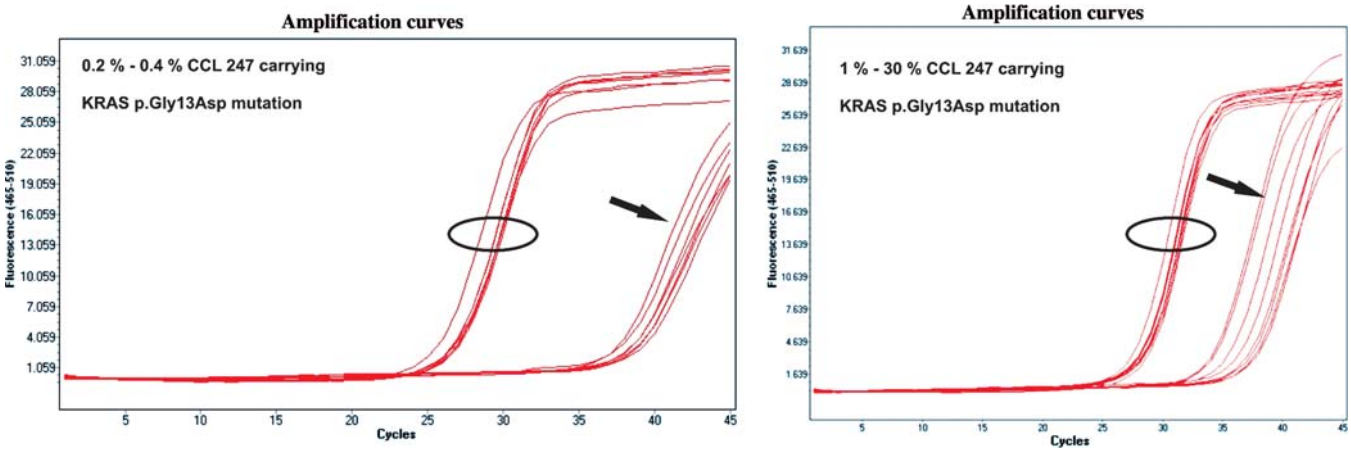


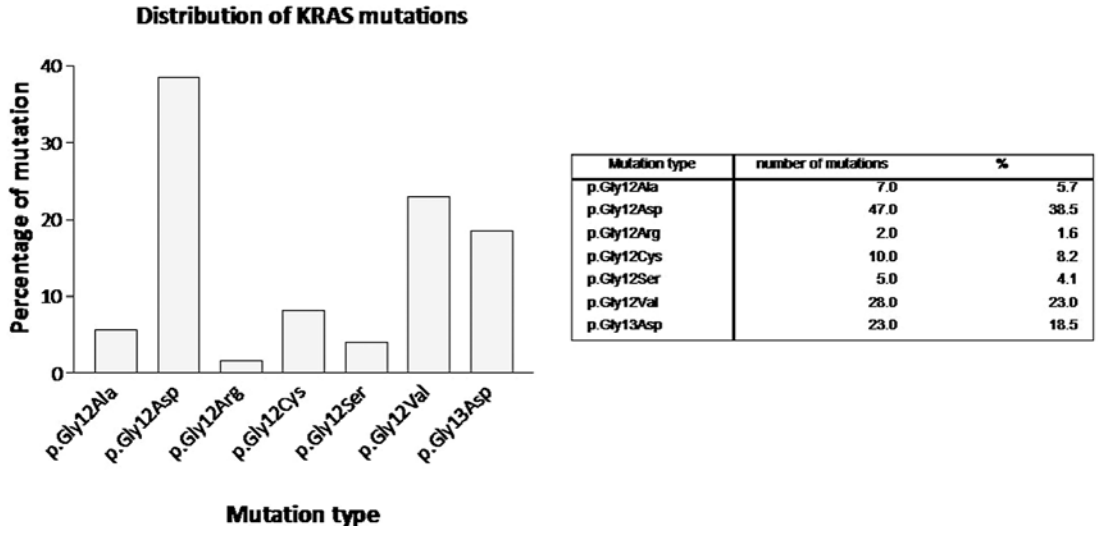
Figure 1. Amplification curves of the mixed samples harboring the p.Gly13Asp mutation of the *KRAS* gene. TheraSreen K-RAS Mutation Kit-amplification curves of DNA isolated from cell mixtures containing non-tumor and tumor cells harboring different proportions of cells carrying *KRAS* p.Gly13Asp mutation. The left picture represents amplification curves of mixed samples with 0.2-0.4% of CCL247 cells harboring the *KRAS* p.Gly13Asp mutation. The circled amplification curves represent the amplification of the mixed standard, while the arrow points at the group of curves representing the amplification of the *KRAS* p.Gly13Asp mutation. As the percentage of CCL247 tumor cells in the sample increases from 1-30% steeper amplification curves and lower threshold cycles are observed (right picture). The circled amplification curves represent the amplification of the mixed standard, and the arrow points at the group of curves representing the amplification of the *KRAS* p.Gly13Asp mutation.

KRAS mutational status and the distribution of KRAS mutations. For the diagnostic purposes a group of 302 samples from 273 patients with metastatic colorectal cancer, treated at the Institute of Oncology Ljubljana, were tested. The genotyping of the *KRAS* gene was not possible for five patients (1.8%) due to poor DNA quality. The test was successfully performed on 98.1% of the tested patients.

Among the successfully tested patients are 146 (54.5%) patients with wt-*KRAS* genotype and 122 (45.5%) patients carrying one of the tested KRAS mutation. The proportional

Table III. The assessment of mutational status for prognostication of the cetuximab therapy outcomes in colorectal cancer patients.

Sample no.	Sample ID	KRAS-status	Response to treatment
1	KR5	wt	Complete response
2	KR12	wt	Partial response
3	KR10	wt	Partial response
4	KR23	wt	Partial response
5	KR22	wt	Partial response
6	KR18	wt	Partial response
7	KR21	wt	Partial response
8	KR1	wt	Partial response
9	KR7	12Ser	Partial response
10	KR9	wt	Stable disease
11	KR2	wt	Stable disease
12	KR16	wt	Stable disease
13	KR20	12Arg	Stable disease
14	KR25	12Asp	Stable disease
15	KR14	12Asp	Stable disease
16	KR4	12Ser	Stable disease
17	KR24	13Asp	Stable disease
18	KR19	13Asp	Stable disease
19	KR17	wt	Progress of disease
20	KR15	wt	Progress of disease
21	KR3	12Asp	Progress of disease
22	KR6	12Asp	Progress of disease
23	KR13	12Asp	Progress of disease
24	KR11	13Asp	Progress of disease
25	KR8	12Val	Progress of disease



Mutation type	number of mutations	%
p.Gly12Ala	7.0	5.7
p.Gly12Asp	47.0	38.5
p.Gly12Arg	2.0	1.6
p.Gly12Cys	10.0	8.2
p.Gly12Ser	5.0	4.1
p.Gly12Val	28.0	23.0
p.Gly13Asp	23.0	18.5

Figure 2. Distribution chart of the seven tested KRAS mutations in the group of tested patients. The proportional distribution of the seven tested *KRAS* mutations among the 122 positive patients.

distribution of the seven tested *KRAS* mutations is presented in Fig. 2.

For 23 patients we obtained and tested a set of two samples; primary tumor tissue sample and metastatic tissue

sample. From these series we were not able to perform the testing for one patient due to the poor quality of the DNA samples; primary tumor and metastatic tissue sample. Of the 22 successfully tested patients 21 patients (95.35%) had matching results between the primary tumor tissue sample and the metastatic tissue sample. Ten (45.5%) of the tested pair samples had a wt-*KRAS* genotype and 12 (55.5%) of the tested pair samples were *KRAS* mutation positive.

KRAS mutational status as a prognosticator of cetuximab therapy outcome. To asses the power of mutational status for prognostication of the cetuximab therapy outcomes we retrospectively tested the DNA samples from 23 patients with metastatic colorectal cancer. *KRAS* status was not known before starting and during treatment. It was determined in December 2008, retrospectively. We tested 25 samples altogether as 2 tumor samples were available for two patients. The patients were treated with cetuximab from 2 to 18 months. In one (1/23) patient complete response was observed. With 6 patients we observed partial response (for two patients two tumor samples were available), 9 patients have stable disease and 7 patients have progressive disease. Six out of seven responders (85.7%) were *KRAS* wt and 5 out of 7 (71.4%) with progressive disease were *KRAS* mutants (Table III). One responder was observed among the 12 patients with a *KRAS* mutation, compared with 6 responders among the 11 patients without *KRAS* mutation (8.3 vs 54.5%, respectively). The observed p-value from the Pearson's Chi-square test was 0.016, which is a statistically significant difference.

Discussion

KRAS status (wild-type or mutated-type) has been proven as a predictor of response to EGFR-targeted therapies for patients with metastatic colorectal cancer (10-14). The *KRAS* mutations are responsible for the synthesis of permanently active *KRAS* protein (23) and are mostly concentrated on 12 and 13 codon of the gene (17). Since activating mutations in the *KRAS* gene are found in 30-40% (17) of colorectal tumors, our laboratory has introduced an RT-PCR-based method (CE-marked diagnostic kit TheraScreen K-RAS Mutation Kit) to identify patients suitable for therapy with anti-EGFR inhibitors.

The assay is created to allow determination of the 7 most frequent mutations in codons 12 and 13 of *KRAS* gene. By the manufacturer's description, the detection level of the assay is 1% of mutated DNA in a background of wild-type DNA (25). We are performing the test in accordance with the recommendations of the European quality assurance program. This includes the identification of the patient (with the indication for testing and patient-specific medical data) by a healthcare professional, data on type of material used for testing and content of tumor cells present in the sample. The used test should be highly sensitive and specific and interpretation of results in the context of the indication for testing (17).

Before the routine daily usage of the TheraScreen K-RAS Mutation Kit for diagnostic purpose, we evaluated the sensitivity and specificity of the method in our laboratory. Firstly we tested the sensitivity of the TheraScreen K-RAS Mutation Kit using different proportions of tumor cells in the sample, to establish the minimum percentage of tumor cells carrying a mutation in a background of non-tumor cells that are required for successful mutation detection. We find this very important, as tumor samples have different amounts of tumor cells present in the sample, especially when the samples are taken after chemotherapy or irradiation therapy. This happens rarely, but the results of the testing must be interpreted even in these cases correctly. From a series of cell mixture samples that was prepared to mimic the heterogeneous tumor tissue, we determined that 2% cells carrying a *KRAS* mutation must be present in the sample for an undisputable detection of mutated signal using the LightCycler Adapt Software. The very same results were obtained when DNA samples with different dilutions were prepared; 2% of mutated DNA was the limit for a positive mutation call by the LightCycler Adapt Software. Our laboratory established detection limit for the method using the LightCycler Adapt Software is 2% of mutated cells. This is to a small extent different from the detection limit declared by the manufacturer; approximately 1% of mutated DNA in the background of *KRAS* wt DNA (25). Indeed, if the Light Cycler Adapt software was not used and the analysis was performed manually the detection limit was increased to 0.2% of mutated cells. The possibility of an error with these dilutions is very high, if we consider the minute concentrations of the cells carrying the *KRAS* mutation that are present in the sample. Therefore, the difference in our laboratory established detection limit of 2% and the manufacturer's detection limit of 1% with the sensitivity interval of 1-2% is practically the same.

To assess the specificity of the method, we evaluated the concordance of our test results for 30 retrospective samples with the results of the pyrosequencing. The pyrosequencing of our samples was performed by the appointed reference laboratory at the Institute of Pathology of the LMU Munich. Our real-time PCR results were in 100% concordance when compared with the pyrosequencing method. Based on this, we gained the *KRAS*-expert laboratory certificate for quality assurance of the molecular-pathological detection of *KRAS* mutations in colorectal cancer in March 2009.

Among the 273 routinely-tested Slovenian patients the ratio of mutated (45.5%) and non-mutated (54.5%) *KRAS* status is slightly elevated compared with the ratio reported by other European countries (10,17,19,20,26-28), but will probably decrease in the favor of non-mutated *KRAS* as the number of tested patients increases with time. The distribution of the seven tested mutations (p.Gly12Ala = 5.7%, p.Gly12Asp = 38.5%, p.Gly12Arg = 1.6%, p.Gly12Cys = 8.2%, pGly12Ser = 4.1%, p.Gly12Val = 23.0% and p.Gly13Asp = 18.5%) among the mutated *KRAS* patients is in concordance with the published data (29,30).

In the group of routinely-tested patients there were 23 patients from whom primary tumor and metastatic tissues were available. This enabled us to investigate the correlation between *KRAS* mutation status of primary tumors and related metastatic tissues. Among the 22 successfully tested pair-matched samples, 21 (95.35%) had a matching result between the primary tumor tissue and related metastatic tissue. We believe the discrepancy between the primary tumor

tissue and the related metastatic tissue in the *KRAS* status could be attributed to the insufficient number of tumor cells in the sample, or lower method sensitivity. Ten of the tested pair samples (45.5%) had a wt-*KRAS* genotype and 12 of the tested pair samples (55.5%) were *KRAS* mutation positive. In contrast to EGFR expression which may differ in primary colorectal tumor tissue and the corresponding metastatic tissue, literature reports high concordance between *KRAS* mutations of primary and metastatic tumors from patients with colorectal carcinoma (31,32). This grade of concordance is important from two aspects. Firstly it suggests that *KRAS* mutations are an early event in colorectal carcinoma pathogenesis and are not essential for metastatic spread of the tumor (31). Secondly, if the primary tumor sample is not available, the evaluation of *KRAS* mutational status can also be performed from a metastatic site with relatively high confidence.

To assess the power of mutational status for prognostication of the anti-EGFR therapy outcomes, we retrospectively tested 23 patients with metastatic colorectal cancer, who were treated with cetuximab from 2 to 18 months. Out of 23 patients 7 patients responded to the therapy. As expected 85.7% of the responders were *KRAS*-wt patients. Among the wt-*KRAS* responders a complete response was achieved only by one patient, while 5 *KRAS*-wt patients showed partial response. Other authors have reported 40-60% of patients with wild-type *KRAS* responding to the treatment (33). This suggests there are other important molecular factors of response that need to be identified. On the other hand one patient from the responders group was a *KRAS* mutant. He responded to all previous chemotherapies with quite a long progression-free survival time, therefore, we conclude the observed response was probably due to another line of chemotherapy, rather than to combination with cetuximab. The group of patients with stable disease gives inconclusive prediction data as the *KRAS* mutational status is mutation negative for 33.3% of the patients. On the other hand among 16 patients out of 23 who did not respond to therapy, 9 patients had stable disease, and 7 had a progression of disease. In the group of non-responders 71.4% of patients had a mutated *KRAS* genotype. This supports the conclusion that the test seems to be highly specific, suggesting complete or partial response to cetuximab is highly unlikely in the presence of a *KRAS* mutation.

We have shown, that the RT-PCR method our laboratory has introduced to discriminate between cetuximab therapy responders and non-responders is efficient, reliable and quickly applicable. The ratio of patients with wt-*KRAS* and mutated *KRAS* status is similar to ratios reported by other authors, so is the distribution of the 7 most common mutations among the mutated *KRAS* patients. Even though the number of responders among the patients with wt-*KRAS* was statistically significantly higher compared to patients with mutated *KRAS* ($p=0.016$), the selection of patients for treatment solely on the basis of *KRAS* status is not perfect. Still, the testing of *KRAS* genotype status is useful to identify patients who will respond to cetuximab, not only for reducing the toxic effects of the therapy for those patients who will not benefit from it, but also to reduce high health care costs.

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