Comparison of the prevalence of *KRAS*-LCS6 polymorphism (rs61764370) within different tumour types (colorectal, breast, non-small cell lung cancer and brain tumours). A study of the Czech population

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Aims. A germline SNP (rs61764370) is located in a let-7 complementary site (LCS6) in the 3'UTR of *KRAS* oncogene, and it was found to alter the binding capability of the mature let-7 microRNA to the *KRAS* mRNA. The aim of the study was to evaluate the frequency of the *KRAS*-LCS6 variant allele in different cancer types that included patients with colorectal cancer (CRC), breast cancer (BC), non-small cell lung cancer (NSCLC) and brain tumour patient subgroups from the Czech Republic. The occurrence of this genetic variant was correlated with the presence of selected somatic mutations representing predictive biomarkers in the respective tumours.

Methods. DNA of tumour tissues was isolated from 428 colorectal cancer samples, 311 non-small cell lung cancer samples, 195 breast cancer samples and 151 samples with brain tumour. Analysis of SNP (rs61764370) was performed by the PCR+RFLP method and direct sequencing. *KRAS, BRAF* and *EGFR* mutation status was assessed using real-time PCR. The status of the *HER2* gene was assessed using the FISH method.

Results. The *KRAS*-LCS6 TG genotype has been detected in 16.4% (32/195) of breast cancer cases (in *HER2* positive breast cancer 3.3%, in *HER2* negative breast cancer 20.1%), in 12.4% (53/428) of CRC cases (*KRAS/BRAF* wild type CRC in 10.6%, *KRAS* mutant CRC in 10.1%, *BRAF* V600E mutant CRC in 18.5%), in 13.2% (41/311) of NSCLC samples, (*EGFR* mutant NSCLC patients in 8%, *EGFR* wild type NSCLC in 12.9%), and 17.9% (27/151) of brain tumour cases. The *KRAS*-LCS6 TG genotype was not significantly different across the studied tumours. In our study, the GG genotype has not been found among the cancer samples.

Conclusions. Based on the findings, it is concluded that the occurrence of the *KRAS*-LCS6 TG genotype was statistically significantly different in association with status of the *HER2* gene in breast cancer. Furthermore, significant association between the mutation status of analysed somatic variants in genes of the EGFR signalling pathway (*KRAS, BRAF, EGFR*) and the *KRAS*-LCS6 genotype in colorectal cancer and NSCLC has not been established.

Key words: *KRAS*-LCS6, *KRAS*, *BRAF*, colorectal cancer, breast cancer, non-small cell lung cancer, brain tumour, genotype, miRNA, polymorphism, FISH

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INTRODUCTION

Germline SNP (rs61764370) has been recently extensively studied in relation to several types of cancer, especially in connection with associated risk, prognosis, and/or treatment response/resistance to therapy[1-11]. The let-7 family of miRNAs has been found to play an important role in tumorigenesis by regulating the expression of multiple oncogenes, including *KRAS*. The *KRAS* (Kirsten rat sarcoma viral oncogene homolog) gene is one of the most frequently mutated oncogenes in human cancer[12], with almost a quarter of different tumour types showing altered functions of the *KRAS* gene. It encodes a protein, which plays a major role in signal transduction and cell proliferation, is a crucial target of let-7 microRNAs (miRNAs) and is downregulated by these miRNAs (ref.[5,13,14]). Let-7 miRNAs downregulate the KRAS protein expression by binding to a specific site in the 3'-untranslated region (3'-UTR) of the KRASmRNA (ref.[13]). Altered expression of the let-7 family of microRNAs is implicated in many human cancers[15-17]. The SNP (rs61764370), also known as *KRAS*-LCS6, is located in the 3'-untranslated region of the *KRAS* oncogene, consists in a T-to-G base change, and it was found to alter the binding capability of the mature let-7 to KRASmRNA, which resulted in both increased *KRAS* expression and reduced let-7 levels[1,4]. Another group of authors has reported that cancer associated non-coding SNP (rs61764370) variant exerts biological effect not through transcriptional modulation of *KRAS* but rather by tuning the expression of microRNA let-7 (ref.[18]).

On the basic of the current evidence and the importance of both miRNA let-7 and *KRAS* oncogenes in human solid tumours, the purpose of our study was to

investigate the frequency of the *KRAS*-LCS6 variant allele in different cancer types that included the following subgroups: colorectal cancer (CRC), breast cancer (BC), non-small cell lung cancer (NSCLC) and brain tumours. Furthermore, the occurrence of this genetic variant was correlated with the presence of selected somatic mutations representing predictive biomarkers in the respective tumour types in Czech population.

MATERIAL AND METHODS

A retrospective analysis was used to test 1085 samples of DNA of tumour tissue from patients in whom *KRAS, BRAF, HER2* and *EGFR* predictive markers (gene status) were analysed in our laboratory between 2008 and 2014 to determine their eligibility for targeted biological treatment. Furthermore, prognostic markers were analysed to determine brain tumour prognosis. The material for the tumour DNA extraction was collected from 428 samples with a diagnosis of metastatic colorectal cancer confirmed histologically, stage III-IV: 277 male subjects (64.7%) aged 35-91 years / 151 female subjects (35.3%) aged 31-94 years; the median age of the CRC cohort was 65 years. The NSCLC group consisted of 311 samples of tumour DNA: 202 male subjects (65.0%) aged 34-99 years / 109 female subjects (35.0 %) aged 32-89 years; the median age of the NSCLC cohort was 67 years. A total of 195 samples of the BC included 121 samples of tumour DNA of invasive ductal carcinoma (62.0%), 15 samples of invasive lobular carcinoma (7.7%), 59 other BC samples (30.3%): 2 male subjects (1.0%) aged 51-77 years / 193 female subjects (99.0%) aged 25-87 years; the median age of the BC cohort was 60 years. A total of 151 samples of tumour DNA in the brain tumour cohort included 117 samples of gliomas, grade I-IV (77.5%), 23 samples of meningiomas (15.2%), 11 samples of other brain tumours (7.3%): 75 male subjects (49.7%) aged 10-77 years / 76 female subjects (50.3%) aged 1-81 years; the median age of the brain tumour cohort was 56.5 years.

The DNA of tumour tissues was isolated from different types of materials: fresh-frozen tissue, formalin-fixed paraffin-embedded tissue and cytology specimens. The commercial kits cobas® DNA Sample Preparation Kit or MagNA Pure Compact Nucleic Acid Isolation Kit (Roche Diagnostics Corporation, GmbH, Mannheim, Germany) were used to isolate the DNA. The DNA quality and quantity was assessed using the NanoPhotometer™ IMPLEN (Implen, GmbH, München, Germany).

Analysis of SNP (rs61764370) (*KRAS*-LCS6) was performed by polymerase chain reaction (PCR) and enzymatic digestion. Twenty microliters of PCR reaction mixture contained: PCR water (Thermo Scientific, Vilnius, Lithuania), PCR Master Mix (2x) (Thermo Scientific, Vilnius, Lithuania), specific primers (0.2 pmol of sense and antisense primers) and DNA. The primers used were as follows: sense, 5′- CCTGAGTAGCTGGGATTACA-3′ and antisense, 5′- GGATACCATATACCCAGTGCCTT-3′ for the *KRAS*-LCS6 polymorphism (amplify a fragment of 232 bp). The PCR conditions were as follows: the initial denaturation was at 95 ˚C for 5 min, followed by 35 cycles of 95 °C for 20 s, 60 °C for 15 s and 72 °C for 10 s; the final amplification was at 72 °C for 5 min, PCR products (232 bp) were checked by electrophoresis on 3% agarose gel.

Following the PCR, the enzymatic digestion was performed with the FastDigest *HinfI* enzyme (Thermo Scientific, Vilnius, Lithuania) for the *KRAS*-LCS6 polymorphism at 37 °C in accordance with the manufacturer's instructions. The reaction was analysed on agarose gel (3%). The gel was stained in an ethidium bromide solution and visualized on Transilluminator UVT-20 (Herolab GmbH, Germany). On the basis of the observed fragments, genotypes were identified as follows: *KRAS*-LCS6 polymorphism; TT genotype: (117 bp + 115 bp), TG (232 bp + 117 bp + 115 bp), and GG (232 bp).

Some positive results were confirmed by direct sequencing. The sequencing reaction was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Foster City, CA, USA). 5 µL of PCR products were cleaned with *Exonuclease I* and FastAP *Thermosensitive Alkaline Phosphatase* (Thermo Scientific, Vilnius, Lithuania) by incubating at 37 °C for 15 min, followed by 15 min at 80 °C, and held at 4 °C. The reaction mix consisted of 3 µL BigDye® Terminator v3.1, 0.5 µL of Big Dye® Terminator Sequencing buffer (5x), 2.5 µL of primer at a final concentration 1.25 pmol/µL and 4 µL of cleaned template in a total volume of 10 µL. The reactions were performed according to the following protocol: 96 °C for 1 min; 25 cycles of 96 °C for 10 s, 50 °C for 5 s, 60 °C for 3 min.

The products of the sequencing reaction were cleaned using the BigDye® XTerminator Purification Kit (Life Technologies, Foster City, CA, USA) in accordance with the manufacturer's instructions. After purification, samples were run on Applied Biosystems® 3130 Genetic Analyzer and analysed using Sequencing Analysis Software version 5.2 (both Life Technologies, Foster City, CA, USA).

The *KRAS* (mutations in codons 12, 13 and 61) and *BRAF* (V600E) mutation statuses were assessed using real-time PCR (cobas® 4800 KRAS Mutation Test, Roche Diagnostics, GmbH, Mannheim, Germany; cobas® 4800 BRAF V600 Mutation Test, Roche Molecular Systems, Inc., Branchbury, NJ, USA respectively). Analysis of 40 mutations within the *EGFR* gene (29 deletions in exon 19, L858R, T790M, G719X including G719S, G719A, G719C; S768I, 5 insertions in exon 20) was performed using the real-time PCR method with cobas® EGFR Mutation Test (CE IVD; Roche) in the NSCLC group. Analysis of the *HER2* gene status was performed by fluorescence in situ hybridisation method (FISH) using the PathVysion HER-2 DNA probe kit (Abbott Molecular).

The statistical analyses were performed using the chi-squared test and Fisher's exact test, the level of significance 5% (when at least one expected value is < 5, Fisher exact tests are recommended rather than chi square). The statistical evaluation was performed using the Stata software, version 13.

RESULTS

We assessed the frequency of the *KRAS*-LCS6 variant allele in 1085 samples of DNA of different cancer types, including CRC, BC, NSCLC and brain tumour. Using the chi-squared test, there was found a significant difference in the distribution of the G-allele (the TG genotype) across individual groups of BC samples divided into two groups on the basis of the *HER2* gene status, ($P = 0.026$). Table 1.

The 95% confidence interval (CI) for occurrence of the TG genotype was determined (Fig. 1).

Further statistical analysis of the *KRAS*-LCS6 (rs61764370) polymorphism in relation to the mutation status of the *KRAS* and *BRAF* genes was performed in patients with colorectal cancer (Table 3). Using he chi-squared test, no significant statistical difference was found in the distribution of the G-allele (the TG/GG genotype) across individual groups of CRC samples divided into three subgroups on the basis of the mutation status of the *KRAS* and *BRAF* genes, ($P = 0.081$).

A statistical analysis of the *KRAS*-LCS6 (rs61764370) polymorphism in relation to the mutation status of the *EGFR* gene was performed in patients with non-small cell lung cancer (Table 4).

The Fisher's exact test indicated no significant statistical difference in the distribution of the G-allele (the TG genotype) across individual groups of NSCLC samples divided into two groups on the basis of the mutation status of the *EGFR* gene, ($P = 0.752$).

DISCUSSION

In selected groups of BC depending on the *HER2* gene status, our results show a significant difference in the distribution of G allele (TG genotype) of the *KRAS*-LCS6 polymorphism. The *KRAS*-LCS6 TG genotype SNP

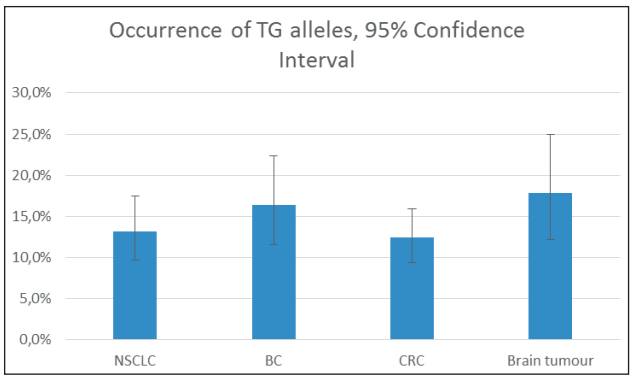

Fig. 1. Distribution of the TG genotype of the *KRAS*-LCS6 (rs61764370) polymorphisms in cancer groups.

Table 1. Distribution of the *KRAS*-LCS6 (rs61764370) genotype in BC patients depending on the *HER2* gene status.

KRAS-LCS6	TT genotype		TG genotype		Total	
	count	%	count	%	count	%
HER2 positive breast cancer	29	96.7	1	3.3	30	100
HER2 negative breast cancer	123	79.9	31	20.1	154	100
chi-squared test, $P = 0.026$						

Table 2. *KRAS*-LCS6 (rs61764370) genotype in experimental groups.

KRAS-LCS6	TT genotype		TG genotype		Total	
	count	%	count	%	count	%
CRC	375	87.6	53	12.4	428	100
NSCLC	270	86.8	41	13.2	311	100
BC	163	83.6	32	16.4	195	100
Brain tumour	124	82.1	27	17.9	151	100
chi-squared test, $P = 0.273$						

Table 3. Distribution of the *KRAS*-LCS6 (rs61764370) genotype in CRC patients depending on the mutation status of the *KRAS* and *BRAF* genes.

KRAS-LCS6	TT genotype		TG genotype		Total	
	count	%	count	%	count	%
KRAS/BRAF wild type CRC	135	89.4	16	10.6	151	100
KRAS mutant CRC	152	89.9	17	10.1	169	100
BRAF V600E mutant CRC	88	81.5	20	18.5	108	100
chi-squared test, $P = 0.081$						

Table 4. Distribution of the *KRAS*-LCS6 (rs61764370) genotype in NSCLC patients depending on the mutation status of the *EGFR* gene.

KRAS-LCS6	TT genotype		TG genotype		Total	
	count	%	count	%	count	%
EGFR mutant NSCLC	23	92.0	2	8.0	25	100
EGFR wild type NSCLC	249	87.1	37	12.9	286	100
Fisher's exact test, $P = 0.752$						

(rs61764370) was found to be 20.1% in the *HER2* negative subgroup compared to 3.3% in the *HER2* positive breast subgroup. These results can implicate that *KRAS*-LCS6 TG genotype SNP (rs61764370) may lead to an alteration in the *HER2* gene expression profile.

These results are inconsistent with the findings published by Cerne[10], who reported no significant association of the TG/GG genotype in group of BC selected depending on the *HER2* status (the authors analysed 506 cases of BC/ 63 cases were *HER2* positive). In addition, they found a positive association of SNP (rs61764370) only in *HER2* positive tumours, high histopathological grade and both associations were detected only in the HRT users.

In the non-selected group of breast cancer samples, the frequency of the TG genotype of SNP (rs61764370) was shown to be 16.4%, and the results are in line with Cerne[10], who found a frequency of 17.2% for the TG genotype in a group of sporadic breast cancers. Furthermore, there is no significant difference in the frequency of the G-allele (TG/GG genotype) *KRAS*-LCS6 polymorphisms either across cancer groups or between cancer and general population reported in study by Chin LJ[1]. Similarly, Paranjape[6] found no difference in the overall frequency distributions of the *KRAS*-variant genotype in cases of breast cancer and controls. In this study, a significant association of the *KRAS*-LCS6 was only found in a group with breast cancer in premenopausal patients with oestrogen/ progesterone-negative tumours.

In the group of colorectal cancers, a total of 428 histologically confirmed samples were examined and the frequency of the TG genotype SNP (rs61764370) was found to be 12.4%. No statistically significant difference in the distribution of the TG genotype, 10.6% *KRAS/BRAF* wild-type, 10.1% *KRAS* mutant, 18.5% *BRAF* mutant was found for individual groups of the CRC samples divided into three subgroups depending on the mutation status of the *KRAS* and *BRAF* genes. Despite an evident tendency of the given allele to be present nearly twice as often in the *BRAF* mutant CRC patients compared to *KRAS/BRAF* wild-type and *KRAS* mutant patients, the chi-squared test indicated no statistically significant difference. The identified frequency of the *KRAS*-LCS6 G-allele (TG genotype) in the *KRAS/BRAF* wild-type group is in line with the data published by Páez[19]. A different result was found in the case of patient stratification to *KRAS* wild-type group and *KRAS* mutant group by Graziano[2]. These authors found significantly greater frequency of the *KRAS*-LCS6 G allele in the *KRAS* mutant group. Previous studies have shown that SNP is a negative prognostic marker in late-stage colon cancer[2], a positive prognostic indicator in early-stage colon cancer[4] and a predictor of response to treatment in colon cancer[2,5,9]. However, the systematic review and meta-analysis of Langevin and Christensen[20] indicated no clear evidence to support an association between the *KRAS*-LCS6 genotype and an overall or progression-free survival among colorectal cancer patients; even after analyses of subgroups stratified by stage and anti-EGFR treatment status. In our analysis, no such result was found and no significant association between the *KRAS* and *BRAF* mutation status and the *KRAS*-LCS6 genotype for the colorectal carcinoma group was established. This is in line with the results published in a Norwegian study[21] and in Sha[22].

In the group of non-small cell lung cancer, a total of 311 histologically confirmed samples were examined. The variant TG genotype was observed in 13.2%. Our results are in line with the results published by Nelson HH[23], whose group of adenocarcinoma patients exhibited a frequency of variant alleles of 14.7%. No statistically significant difference in the distribution of the G allele (TG genotype) was found between individual groups of the NSCLC samples divided into two subgroups depending of the mutation status of the *EGFR* gene.

In a group of patients with brain tumours, constituting a heterogeneous group of 151 histologically confirmed samples, the TG genotype of SNP (rs61764370) was detected in 17.9%. Even in this group, no significant difference in comparison with other types of tumour was found. In the literature available to date, no study has been published so far making a comparison with a similar group of brain tumours possible.

The occurrence of this SNP varies geographically across the global population. This follows from the ALFRED (The ALlele FREquency Database) expected heterozygosities (rs61764370) for persons of European descent[24] are 0.05-0.25. Chin LJ[1] reported that in the general population the frequency of the G-allele of *KRAS*-LCS6 is 5.8% and about 7.6% in populations of European descent, based on the genotyping of 2.433 samples (representing 46 geographic populations).

CONCLUSION

The determination of risk factors for cancer provides improved tools for its diagnosis and treatment. Because of the importance of both miRNA let-7 and *KRAS* oncogenes in human solid tumours and their potential use

as a prognostic/predictive marker, the frequency of the G-allele of the SNP (rs61764370) *KRAS*-LCS6 in different types of solid tumours was evaluated. Based on the findings, it is concluded that there is a significant difference in the *KRAS*-LCS6 TG genotype associated with gene *HER2* status in breast cancer. This result can implicate that the *KRAS*-LCS6 G-allele (TG/GG genotype) may lead to alteration in *HER2* gene expression profile (could be a potential genetic marker of a developing *HER2* negative breast cancer). A further analysis of BC patients is necessary to confirm these results.

However, no significant difference in the occurrence of the *KRAS*-LCS6 G-allele (TG/GG genotype) across the studied tumour types (CRC, NSCLC, BC and brain tumour) was found.

ABBREVIATIONS

CRC, Colorectal cancer; NSCLC, Non-small cell lung cancer; BC, Breast cancer; PCR, Polymerase Chain Reaction; RFLP, Restriction Fragment Length Polymorphism; SNP, Single Nucleotide Polymorphism; EGFR, Epidermal growth factor receptor; DNA, Deoxyribonucleic acid; HRT, Hormone replacement therapy; FISH, Fluorescence in situ hybridisation.

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