Clinical significance of *HRAS* and *KRAS* genes expression in patients with non–small-cell lung cancer.

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Abstract

Background: The *RAS* family protooncogenes, including *KRAS*, *NRAS* and *HRAS*, encode proteins responsible for the regulation of growth, differentiation and survival of many cell types. The *HRAS* and *KRAS* oncogene mutations are well defined, however, the clinical significance of *RAS* expressions in non–small-cell lung cancer (NSCLC) is still uncertain.

Methods: A total of 39 whole blood samples of NSCLC (the investigated group), collected at three points of time: at the time of diagnosis, 100 days and one year after the surgery, were included in this study. *HRAS* and *KRAS* genes mRNA expression were assessed using quantitative real time-polymerase chain reaction techniques.

Results: Increased relative *HRAS* mRNA levels were found significantly more frequently in the group of smokers (p=0.008). Patients with squamous cell carcinoma subtypes of NSCLC were more likely to show an overexpression of *HRAS* gene, but not statistically significant (p=0.065). The median overall survival was significantly better in the group with a higher *HRAS* gene expression (p=0.012). No statistically significant associations were found for the expression of *KRAS* with any clinicopathological parameters within the study. There were no differences between the relative *HRAS* and *KRAS* genes expression levels in blood samples taken from the same patients during the 3 observation points.

Conclusion: The potential associations between high *HRAS* levels, smoking status and histological type of cancer as well as overall survival were observed, which emphasizes the need for further study of the *RAS* family. Therefore, subsequent research involving larger numbers of patients and a longer follow-up, as well as multicenter study are necessary to confirm our findings.

Background

Lung cancer is a significant worldwide health problem, accounting for more than 1.8 million new cases estimated in 2012 and 1.6 million cancer-related deaths annually[1]. The two main types are: small cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). NSCLC comprises about 85% of all lung cancer cases, which have totally different etiology and treatment options[2]. NSCLC mainly includes squamous cell carcinoma, adenocarcinoma and large cell carcinoma. When patients are diagnosed in the early stages of NSCLC, the survival rates are relatively higher after a surgical resection. In patients with an untreated metastatic NSCLC present an overall survival (OS) rate in one year of only 10%, with a median survival of around 4 to 5 months[1].

Over the years, many genes/proteins have been discovered to play a key role in carcinogenesis[3]. RAS proteins are among the most commonly mutated cancer causing agents in humans and remain a difficult pharmaceutical target[4]. Members of the RAS family are low molecular weight monomeric, GTP-binding proteins that play a crucial role as a major component of cellular networks controlling various signaling pathways: growth regulation, proliferation, survival, differentiation, adhesion, cytoskeletal rearrangements, motility and cell survival[5, 6]. Previous research have improved the understanding of the

structure, processing and signaling pathways of RAS in cancer cells and opened up new avenues for inhibiting RAS function[4, 5]. Abnormally activated RAS proteins regulate the function of major signaling pathways involved in the initiation and development in one-third of human cancers [3, 7, 8]. RAS proteins act as a cellular switch that is turned on by extracellular stimuli, resulting in the transient formation of an active, GTP-bound form of RAS that activates various signaling pathways which regulate basic cellular processes.

RAS genes family comprise of three *RAS* protooncogenes such as *HRAS*, *KRAS* and *NRAS* encoded four isoforms: *NRAS, HRAS, KRAS4A* and *KRAS4B*, the last two arising from alternative splicing variants of the *KRAS* gene[4]. The irreversible changes in the genetic content of a cell are a major cause of carcinogenesis as it can modulate both gene expression and the function of proteins involved in the regulation of cell growth and differentiation[3, 5]. *RAS* mutations have quite different patterns and vary with cancer. An oncogenic alteration in *KRAS* gene is the most frequent in pancreatic cancer, colorectal cancer and lung cancer, while mutated *HRAS* is the most common in dermatological, head and neck cancers. The *NRAS* mutations are often detected in hematological malignancies [3]. Since *RAS* genes are among the earliest mutated genes in various cancers, understanding how these mutation patterns arise can help not only understand how the cancer begins, but also the factors influencing the event that impact prevention and cancer treatment[7]. Till now, two general approaches have been explored for inhibiting RAS activity with small molecules: compounds that bind directly to RAS protein and inhibitors of the enzymes involved in the post-translational modifications of RAS [4].

The molecular development of NSCLC is initiated by the activation of oncogenes or the inactivation of tumor suppressor genes. Mutation of *KRAS* gene in lung cancer is more frequent than *NRAS* and *HRAS* and is often associated with poor prognosis and worse therapeutic outcome. Lung adenocarcinoma, the most common histological subtype of non-small-cell lung cancer (NSCLC), often carries a *KRAS* mutation with 20–50% frequency, followed by squamous cell carcinoma, subtype of NSCLC [3].

The present study was designed to determine the potential significance and the clinical relevance of two *RAS* gene family isoforms (*KRAS* and *HRAS*) and their mRNA expression levels in the group of non-small-cell lung cancer patients. Majority of previous scientific findings have focused on the mutation profiles of NSCLC patients or evaluation genes expression in lung cancer tissue. This research was aimed at analysing the relative levels of *KRAS* and *HRAS* genes mRNA expression in whole blood samples, which is an innovative approach, at three points of time during the patients' observation.

Methods

Materials

This retrospective study included 39 patients (32 male and 7 female) with NSCLC from the Department of Thoracic Surgery of the Medical University of Lodz, N. Copernicus Regional Specialist Hospital in Lodz, Poland. Peripheral blood samples were taken at three points of time during the disease and treatment process. The investigation was in accordance with the Declaration of Helsinki, the Good Laboratory

Practice rules and was approved by the Ethical Committee of the Medical University of Lodz (No: RNN/87/16/KE, RNN 85/20/KE). All patients provided a written informed consent before their inclusion in the study.

RNA was isolated from 39 samples at time of the diagnosis of cancer, 37 samples 100 days after the surgery and 26 samples one year after the surgery. These differences between the number of groups resulted from causes such as the death of patients between consecutive examination or losing patients from observation.

RNA isolation

RNA was isolated from whole blood samples using a Total RNA Prep Plus Minicolumn Kit *(A&A Biotechnology, Poland)* according to the manufacturer's protocol. The purity and the concentration of RNA in samples were measured nanospectrophotometrically. The concentration in all samples derived from patients suffering from NSCLC was adjusted to 0.05 µg/µl. Until the analysis, the RNA samples were stored at − 80 °C.

Reverse transcription

RNA samples were transcribed into cDNA using a High Capacity cDNA Reverse Transcription kit *(Applied Biosystems, USA)*, according to the manufacturer's instructions. The thermocycling reverse transcription parameters were as follows: 25˚C for 10 min, then 37˚C for 120 min and 85˚C for 5 min. Until the analysis, the cDNA samples were stored at − 20 °C.

A housekeeping *GAPDH* gene, encoding glyceraldehyde-3-phosphate dehydrogenase, was used as reference gene. The samples with the presence of PCR product for the *GAPDH* gene (145 bp) were used in the following analysis.

PCR amplification

The qualitative assessment of the obtained cDNA was performed by means of PCR amplification of the *KRAS* and *HRAS* genes JumpStart REDTaq ReadyMix *(Sigma-Aldrich, USA)*.

PCR reactions were performed in a volume of 20 µL, components of the PCRs for both genes were the same: 0.7 µl of each primer at a concentration of 10 µM for *KRAS* (forward GCCTGCTGAAAATGACTG, reverse TCCTGTAGGAATCCTCTATTG) and for *HRAS* (forward CACGGAAGGTCCTGAGGGG, reverse GCCTGGCCCCACCTGTG); 10 µl of Reaction Mix, 0.2 µl of 25 mM magnesium chloride solution and 1 µl of template.

PCR amplification was set at an initial 96 °C for 1 min and then, for both of genes, 34 cycles of 94 °C for 50 s, 57 °C (*KRAS*) and 58 °C (*HRAS*) for 50 s, 72 °C for 1 min, and the final extension at 72 °C for 7 min. The annealing temperature was standardized by gradient PCR at 56 °C–61 °C for both genes. After the reaction, PCR products 134 bp for *KRAS* and 276 bp for *HRAS* were evaluated during electrophoresis in 2% agarose gel to assess the quality of cDNA.

Real-time PCR

Relative mRNA expressions level of *HRAS* and *KRAS* genes were analysed using a quantitative real time-polymerase chain reaction (qRT-PCR) on The CFX Connect Real-Time PCR Detection System *(Bio-Rad Laboratories).* The reaction mixture contained: 5 µl iTaq Universal SYBR Green Supermix *(Bio-Rad Laboratories)*, 0.3 µl of a 10 µM solution of each primer specific for the investigated genes, 3.4 µl of distilled water and 1 µl of template up to 10 µl final volume. The qRT-PCR reactions for each sample were performed in triplicates. The same primer set was used for the qualitative analysis. A negative control without template was also added in a triplicate. The real-time PCR conditions were as follows: 10 min 95 °C primary denaturation, 40 cycles of 95 °C for 30 s, 58 °C for 1 min and 72 °C for 1 min. The mean of the obtained Ct values for three genes was counted.

The ΔΔCt method was used to calculate relative expression level changes in genes. The mean Ct values for the *KRAS* and *HRAS* genes and *GAPDH* as a reference gene achieved for all tested samples were assumed as a calibrator. In subsequent reactions, specific amplification was verified using the melting curve analysis.

Statistical analysis

The statistical analysis was performed using the Statistica 13.1. *(StatSoft, Inc., Tulsa, OK, USA*).

The obtained quantitative data were verified with a normal distribution using the Shapiro-Wilk test. Due to the lack of conformity with normal distribution, a comparative statistical analysis was performed using the nonparametric U-Mann Whitney test. The overall survival was assessed using the Kaplan–Meier method and a comparison was carried out using a log-rank significance test. The Friedman test and Kendall's W test were used to verify the differences between the relative level of *KRAS* and *HRAS* genes expression between samples collected at three points of time. In all conducted tests a p value of < 0.05 was assumed as statistically significant.

Results

Patient characteristics

The investigated group consisted of whole blood samples collected at the 3 points of time: 39 samples at time of diagnosis, 37 samples at 100 days after the surgery and 26 samples taken one year after diagnosis.

A total of 23 Polish patients with squamous cell carcinoma subtypes of NSCLC and 16 Polish patients with adenocarcinoma subtypes of NSCLC were enrolled. Investigated group included 32 (82%) men and 7 (18%) women. There were 14 (36%) non-smokers and 25 (64%) cigarette smokers, the median age was 68 years (ranging between 54–82 years). In this group 29 (74%) were at the G2 tumour histological grade, 8 (21%) at G3 tumour grade and 3 (8%) at G1.

Sex, smoking status, histological type and pathologic stage distribution of the investigated group diagnosed as non-small-cell lung cancer with histopathological examination as well as white blood cells,

red blood cells and platelet count are listed in detail in Table 1. In addition, the Neutrophil to Lymphocyte Ratio (NLR), the Lymphocyte to Monocyte Ratio (LMR) and the Platelet to Lymphocyte Ratio (PLR) were calculated.

Table 1
Clinicopathological and laboratory characteristics of patients with NSCLC.

Parameter	N	%	Median	Range
Total	39			
Sex				
Male	32	82		
Female	7	18		
Smoking				
Smoker	25	64		
Non-smoker	14	36		
Histological type of cancer				
Adenocarcinoma	16	41		
Squamous cell carcinoma	23	59		
Grade of histological malignancy [G]				
G1	3	8		
G2	29	74		
G3	8	21		
TNM stage				
IA1	1	3		
IA2	7	18		
IB	11	28		
IIA	7	18		
IIB	5	13		
IIIA	8	21		
WBC ($x10^9$/l)			9.65	6.02–19.39
RBC ($x10^{12}$/l)			4.61	3.54–5.84
PLT ($x10^9$/l)			299	167–961
NLR			3.88	0.97–20.39
LMR			2.2	0.62–5.87

Parameter	N	%	Median	Range
PLR			154	61–976
Abbreviations: NLR, Neutrophil to Lymphocyte Ratio; LMR, Lymphocyte to Monocyte Ratio; PLR, Platelet to Lymphocyte Ratio; TNM, tumor-node-metastasis; WBC, white blood cells; RBC, red blood cells; PLT, platelets				

Qualitative expression of the KRAS and HRAS genes in blood patients with lung cancer

All of the samples revealed the presence of *GAPDH* gene expression using quantitative PCR. 39 samples demonstrated the presence of *KRAS* and *HRA*S gene expression at the time of the diagnosis. 100 days after the surgery, 25 out of 37 samples revealed a qualitative *KRAS* gene expression and all of them presented a qualitative *HRAS* gene expression. One year after the surgery, 17 out of 26 samples were *KRAS* positive and 21 out of 26 samples were *HRAS* positive. Other samples showed no expression of the investigated genes at the second and third point of time during observation. The quantitative analysis indicated that genes expression of the tested *KRAS* and *HRAS* relative to the reference gene *GAPDH* were highly varied.

Quantitative expression of the KRAS and HRAS genes in blood patients with lung cancer

All samples with the presence of *GAPDH* gene expression were included into the quantitative analysis. Relative *KRAS* and *HRAS* mRNA expression levels was determined using qRT-PCR in all 39 samples that were available for the analysis. At the time of the diagnosis, the median *KRAS* mRNA expression was 1.055 (ranging between 0.075–5.339), while the median *HRAS* mRNA expression was 1.153 (ranging between 0.122–4.376). The associations of median relative *KRAS* and *HRAS* mRNA expression (R-value) with clinicopathological features of NSCLC patients are summarized in Table 2.

Initially, the examined population was divided into two groups according to age: 22 people under 68 years old and 17 people aged over 68 years of age. No statistically significant differences were observed between relative *KRAS* and *HRAS* gene expression levels and patients age ($p = 0.403$ and 0.343, respectively).

No statistical differences were also found for relative *KRAS* and *HRAS* gene expression levels between women and men ($p = 0.756$ and 0.596, respectively).

The lung cancer cohort was divided into the subgroup of tobacco smokers (N = 25) and non-smokers (N = 14). Patients who were smokers had a significantly higher median relative for *HRAS* mRNA expression ($p = 0.008$). Data is summarized in Fig. 1. Interestingly, such an association was not found for the relative *KRAS* mRNA expression.

Then, the investigated group was divided according to the histological type of cancer. Although the higher relative level of *HRAS* gene expression was observed in the group of patients with squamous cell

carcinoma subtypes of NSCLC (N = 23) than in the group with adenocarcinoma (N = 16), this difference was not statistically significant ($p = 0.065$; Fig. 1).

There were no associations between the relative *KRAS* mRNA expression and histological type, TNM stage, histological tumour grade or chemotherapy treatment. Similarly, no statistically significant correlations were found for TNM, histological tumour grade or chemotherapy treatment and the expression of *HRAS* mRNA.

Table 2 Relative expression levels of *KRAS* (A) and *HRAS* (B) mRNA in NSCLC patients, compared to clinicopathological and demographical features.

A)

		Relative *KRAS* expression level				
		N	**Median**	**Minimum**	**Maximum**	**p-value**
All cases		39	1.0550	0.0753	5.3395	
Cigarettes	No	14	0.9968	0.0753	2.6566	0.849
	Yes	25	1.1025	0.4669	5.3395	
Gender	Women	7	1.0137	0.5820	2.6566	0.756
	Men	32	1.0787	0.0753	5.3395	
Age	<= 68	22	1.0787	0.2558	5.3395	0.403
	> 68	17	1.0137	0.0753	2.0498	
Histological type of cancer	Squamous	23	1.0137	0.4669	3.1129	0.558
	Glandular	16	1.0787	0.0753	5.3395	
TNM stage	IA1 and/or IA2 and/or IB	19	1.0137	0.0753	5.3395	0.603
	II A and/or II B and/or IIIA	20	1.0787	0.4669	2.6566	
Grade of histological malignancy [G]	G1 and/or G2	31	1.1025	0.0753	5.3395	0.339
	G3	8	0.9094	0.2558	2.6566	
Chemotherapy	No	26	0.9968	0.0753	5.3395	0.205
	Yes	13	1.1025	0.8031	2.6566	

B)

		Relative *HRAS* expression level				
		N	Median	Minimum	Maximum	p-value
All cases		39	1.1532	0.1223	4.3762	
Cigarettes	No	14	08188	0.1223	1.8282	0.008
	Yes	25	1.3125	0.3291	4.3762	
Gender	Women	7	0.9777	0.1397	1.9223	0.596
	Men	32	1.1868	0.1223	4.3762	
Age	<= 68	22	1.2519	0.1397	4.3762	0.342
	> 68	17	0.9777	0.1223	1.9636	
Histological type of cancer	Squamous	23	1.2358	0.3291	4.3762	0.065
	Glandular	16	0.8735	0.1223	1.8282	
TNM stage	IA1 and/or IA2 and/or IB	19	1.1532	0.1223	1.9636	0.683
	II A and/or II B and/or IIIA	20	1.1260	0.1397	4.3762	
Grade of histological malignancy [G]	G1 and/or G2	31	1.1532	0.1223	2.5474	0.903
	G3	8	1.1747	0.1397	4.3762	
Chemotherapy	No	26	1.1868	0.1223	4.3762	0.333
	Yes	13	0.8922	0.1397	1.7389	

Survival analysis

To carry out a survival analysis, the examined group was divided into two subgroups according to the median relative value of relative *KRAS* and *HRAS* genes expression levels (1.055 and 1.153, respectively) in the entire group. The subgroup described as “low” was composed of cases showing the expression level lower or equal than the median. On the other hand, cases presenting expression higher than median were categorized as “high”.

The comparison of the two subgroups of relative *HRAS* gene expression, based on a 1-year period of the patients’ observation, revealed a statistically significant difference in the survival time probability ($p = 0.012$). Interestingly, patients with the high (362 days) relative *HRAS* gene expression level showed a longer survival time than patients with the low (305 days) relative *HRAS* gene expression.

Kaplan-Meier curve for overall survival did not display a statistically significant association between survival and relative *KRAS* gene expression ($p = 0.129$). Nevertheless, in the group with the low relative *KRAS* gene expression, an average length of survival (268 days) was shorter in relation to the group with a high relative *KRAS* gene expression (391 days) (Fig. 2).

Comparison of the changes between the relative level of KRAS and HRAS genes expression in blood patients with lung cancer during a one-year observation at 3 points of time.

Levels of investigated genes were checked in the duration of the disease which was clinically important. The changes of relative levels of relative *KRAS* and *HRAS* genes expression were assessed at three points of time (Fig. 3). There were no statistically significant differences between these groups ($p = 0.766$ for *KRAS*, $p = 0.766$ for *HRAS*).

Discussion

In the present study, relative *KRAS* and *HRAS* genes expression levels were retrospectively evaluated in 39 whole blood samples collected from patients with NSCLC at three points of time (at the time of diagnosis, 100 days and one year after the surgery) using a real-time PCR method. The most important observations from the presented work were that overexpression of *HRAS* gene has occurred more frequently in smokers and there was a tendency to the higher expression of the *HRAS* gene in patients with squamous cell carcinoma subtypes of NSCLC. Moreover, a higher relative expression level of *HRAS* gene was associated with the increased probability of survival. These findings could lead to improving the diagnosis of NSCLC as well as have a clinical implication for a molecular targeted therapy. The data from the current study suggest that the expression of *HRAS* mRNA is worth further evaluation as a prognostic biomarker in lung cancer.

The three members of the *RAS* gene family (*HRAS, KRAS* and *NRAS*) are well-known because of their common oncogenic activation in human tumors, therefore they act as protooncogenes. The expression of *HRAS, KRAS* and *NRAS* genes is frequent in different species, although there are specific variations of expression levels, depending on the tissue and the developmental stage under study[9].

The majority of reported data focus on the frequency of *RAS* genes mutations. They indicate that *RAS* genes are often mutated in different types of tumor and participate in their proliferation apoptosis, migration and the differentiation of the cells. The *KRAS* mutation is common in smoking lung adenocarcinoma patients with frequency between 12% and 36%. *KRAS* mutation frequency in squamous cell lung carcinoma in smokers is 2.7%. The *HRAS* mutations are detected very rarely in lung cancers (< 1%) [10–12]. The prognostic value of *KRAS* and *HRAS* expression has been evaluated in various types of cancers so far, but only few studies indicated the association between the *RAS* family overexpression or mutation and the high degree lesions. However, the expression of these oncogenes in different types of cancer tissue samples was mostly analysed using immunohistochemistry. There are only few studies dedicated to *KRAS* and *HRAS* expression at mRNA level in lung cancer tissue [13–16]. To our knowledge, there is no research focusing on expression *RAS* genes determination in the whole blood assessed in

Polish patients with NSCLC. Due to the limited data dedicated to the evaluation of the *RAS* genes expression in lung cancer, in current study the assessment of relative *KRAS* and *HRAS* gene expression level was performed. This research was aimed at considering the potential clinical usefulness *KRAS* and *HRAS* gene as tumour markers playing a role in the diagnosis (the differentiation between malignant and benign disease) as well as in progression. The presented study showed that the relative expression of *HRAS* gene in patients with NSCLC was positively correlated with tobacco smoking. Taking into account the clinical characteristics more patients were smokers, which correlates with the data from literature indicating that smoking is strongly associated with lung cancer risk. These data suggest that smoking and overexpression of *HRAS* gene are important risk factors in lung cancer arise. Our results are similar to Krishna et al. findings which point out that the frequency of *HRAS* positive expression was higher in patients with oral squamous cell carcinoma who were smokers[15]. Interestingly, the relative expression level of *HRAS* gene was shown to be higher in the squamous lung cell carcinoma than in glandular subtypes, indicating that *HRAS* expression may be regulated developmentally and differentially. Unfortunately, it is not possible to compare these findings with other research due to lack of evidence from previous study focusing on this issue. Another explanation for the significant differences in the *HRAS* gene expression level but not *KRAS* in NSCLC patients, seems the fact that oncogenic alterations in *KRAS* are more frequent in patients with lung malignancies[17], which is why changes in *KRAS* expression might not be detectable. On the other hand, Liang W. et al. showed that the expression of *KRAS* mRNA and protein was significantly increased in NSCLC compared the non-tumour tissues ($p = 0.03$ and $p = 0.018$, respectively). Moreover, the expression of KRAS protein was associated with tumour stages and also occurred more frequently in ever-smokers ($p = 0.002$)[18]. Zhou et al. found that *KRAS* overexpression was common in acute myeloid leukaemia patients and was associated with shorter overall survival [19]. In other study, Sugita et al. showed that *HRAS* expression levels were significantly upregulated in bladder cancer cell lines[20]. In this study, no significant association between the *KRAS* mRNA expression and clinicopathological parameters was observed. In current study, overall survival analysis indicated that a higher primary *HRAS* gene expression level was associated with longer survival times. The OS of NSCLC patients with higher *KRAS* expression levels was longer than in patients with lower *KRAS* expression levels, albeit not significantly. These findings could have some significance as prognostic factors in lung cancer cohort. In contrast to the presented result, Wan et al. displayed an association between a lower *HRAS* expression level with longer overall survival in cutaneous melanoma[21]. Furthermore, An et al. showed *RAS* as an independent predictor of overall survival in patients with lung cancer who were treated with bevacizumab and chemotherapy. They revealed that a lower *RAS* expression level was associated with longer survival time [14]. Similarly, Chen et al. revealed *KRAS* overexpression in patients with colorectal cancer and the high expression of *KRAS* predicted poor treatment outcomes in patients. However, they analyzed the protein level of KRAS in tissue samples using immunohistochemistry[22]. In spite of the fact that *RAS* isoforms share related downstream pathways, their posttranscriptional regulation may differ from each other[21]. Our study suggests that regulation of *HRAS* in non-small-cell lung carcinoma vary from that of *KRAS*. RAS isoform differences have been identified at the level of protein translation and provide a potential explanation for the highest frequency of *KRAS* mutation in cancers. *KRAS* DNA coding sequence has a high frequency of rare codons, causing

poor KRAS protein translation and expression, which is in contrast to *HRAS*. It has been suggested that the overexpression of *HRAS*, but not *KRAS*, induces senescence. Therefore, a cell with mutated *KRAS* will persist to allow succeeding genetic events to promote tumor progression [23], which could be an explanation as for why we did not notice statistically significant changes in the *KRAS* gene expression. To observe the possible dynamic changes of *KRAS* and *HRAS* expression in NSCLC patients at different clinical stages, *RAS* expression levels were assessed at three points of time during the follow-up studies. Neither *KRAS* nor *HRAS* expression tended to fluctuate for the time of a 1-year observation compared to newly diagnosis time. Both *KRAS* and *HRAS* gene expression were not significantly increased or decreased between the first assessment, 100 days and one year after the diagnosis. Consequently, they could not be used as markers of the disease progression or the effectiveness of the therapy. As Liang et al. have noticed, the differential expression of genes in NSCLC suggests the presence of a complex regulatory network involving tumor suppression and oncogenic expression[18]. Therefore, it is worth conducting a further investigation. We are aware of the limitations of the current research, such as small an investigated group from a single institution, using only whole blood samples to assess the mRNA expression levels of *RAS* genes and then comparing their connections, but the results described in this paper are preliminary. Future studies will include a larger cohort of NSCLC patients with longer follow-ups and multicenter study to verify the obtained findings. The next step of the analysis will be extended to the evaluation of *KRAS* and *HRAS* genes expression, both in sample tissue and the control group, and then statistical reanalysis.

Conclusions

Overexpression of *HRAS* gene was observed in the group of smokers and tended to be more presumable in squamous cell lung cancer. Therefore, it could be the potential target in molecular treatment of patients with clinicopathological features mentioned above. This study did not reveal statistically significant differences between the relative expression level of *KRAS* and *HRAS* genes in NSCLC patients at three points of time during the observation, thus it is not possible to use them as markers of the disease progression. Nevertheless, a higher relative expression level of *HRAS* gene at the time of diagnosis was associated with the increased overall survival. It might be useful to measure the primary *HRAS* gene expression level in order to assess the probability of patients' survival. This study emphasized the role of *HRAS* and *KRAS* gene expression level and their heterogeneity in non-small-cell lung cancer cases. It may improve our knowledge for a better diagnosis, identifying patients at high risk of a disease and selecting those who would benefit from a molecular targeted therapy.

List Of Abbreviations

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

HRAS: Harvey rat sarcoma viral oncogene homolog

KRAS Kirsten rat sarcoma viral oncogene homolog

NLR: Neutrophil to Lymphocyte Ratio

LMR: Lymphocyte to Monocyte Ratio

NRAS: neuroblastoma RAS viral oncogene homolog

NSCLC: non–small-cell lung cancer

OS: overall survival

PLR: Platelet to Lymphocyte Ratio

PLT: platelets

RBC: red blood cells

TNM: tumor-node-metastasis

WBC: white blood cells

Declarations

Ethics approval and consent to participate

The investigation was in accordance with the Declaration of Helsinki, the Good Laboratory Practice rules and was approved by the Ethical Committee of the Medical University of Lodz (No: RNN/87/16/KE, RNN 85/20/KE). All patients provided a written informed consent before their inclusion in the study.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

Authors' contributions

MP and EB designed the study and developed methodology, MŻN and MP analyzed the data, MP performed experiments, MP, EB, KM drafted the manuscript, IZ and MŁ organized data and constructed databases. All authors read and approved the final manuscript.

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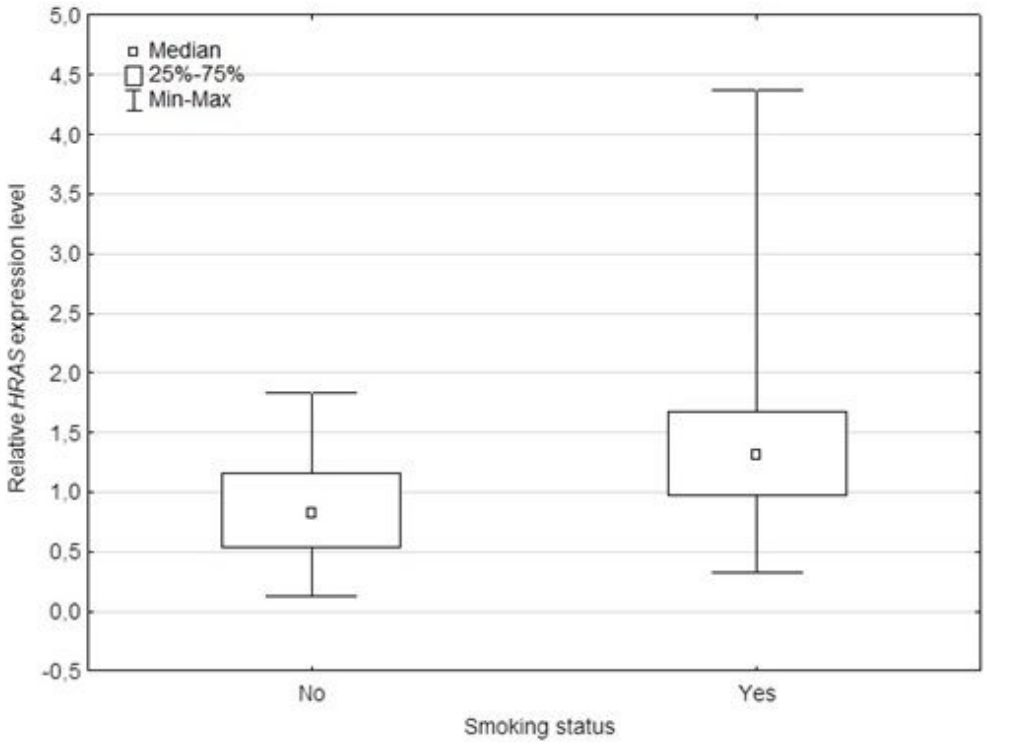
Figures



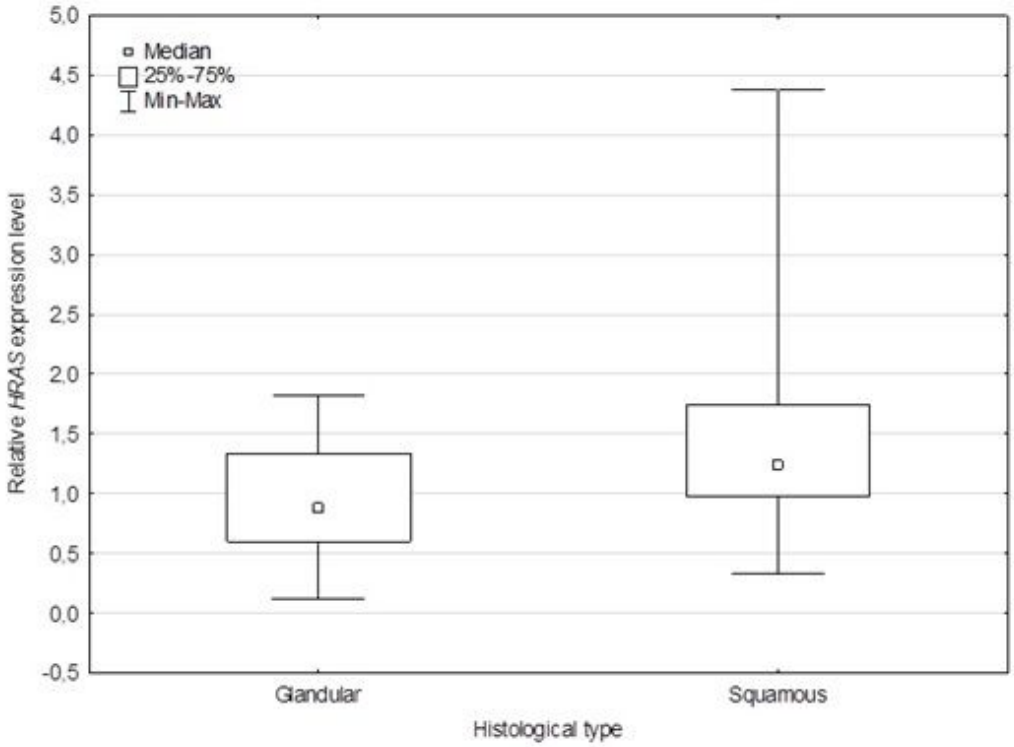


Figure 1

Relative HRAS gene expression level in non-small-cell lung cancer cases. Figure A shows the dependence of the relative HRAS gene expression level in relation to the smoking status of patients included in the study. Figure B shows the relative HRAS gene expression level depending on the histological type of cancer. The data plots for each of the study groups represent the median value along with the minimum and maximum values and the lower and upper quartiles. Statistically significant differences were

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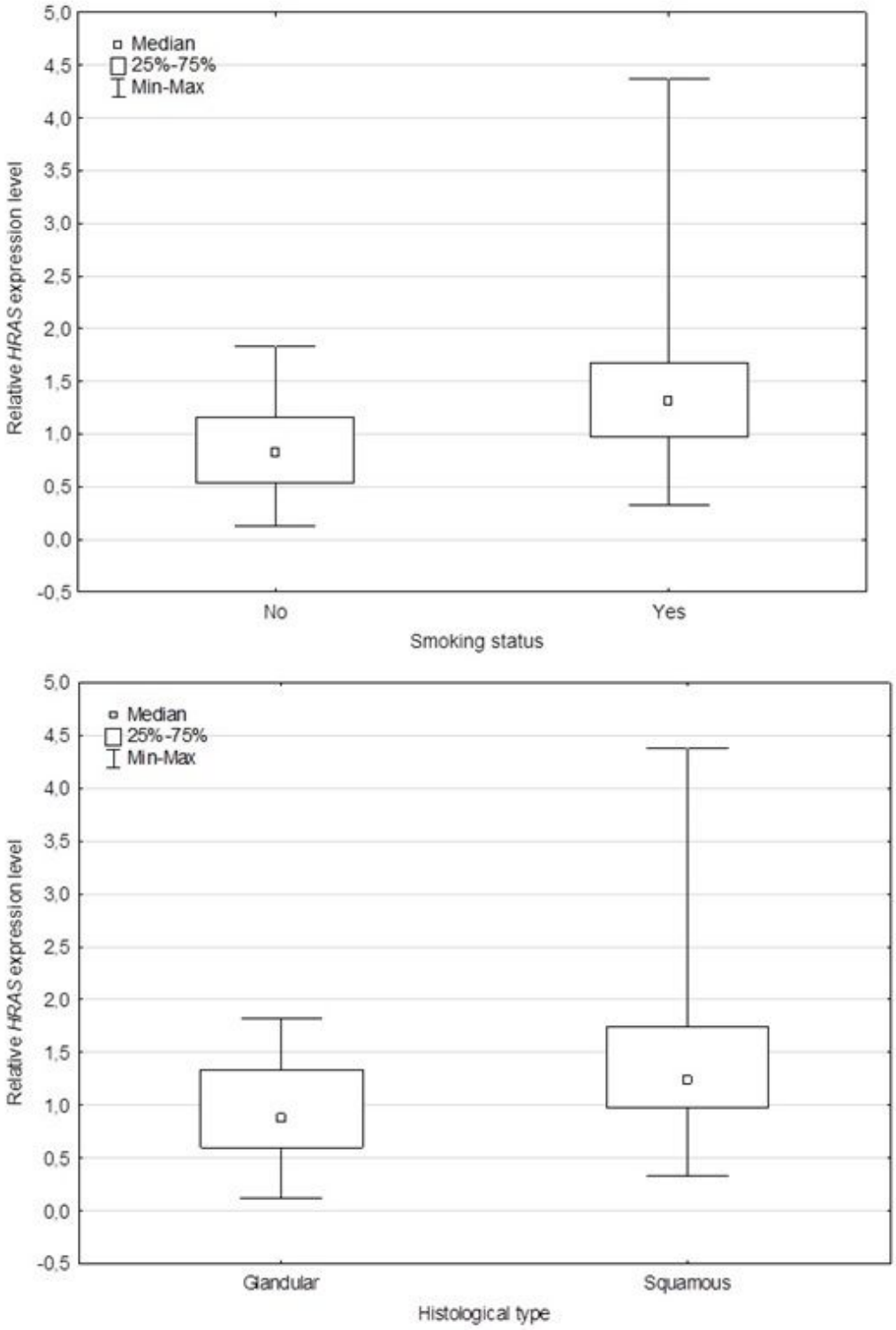

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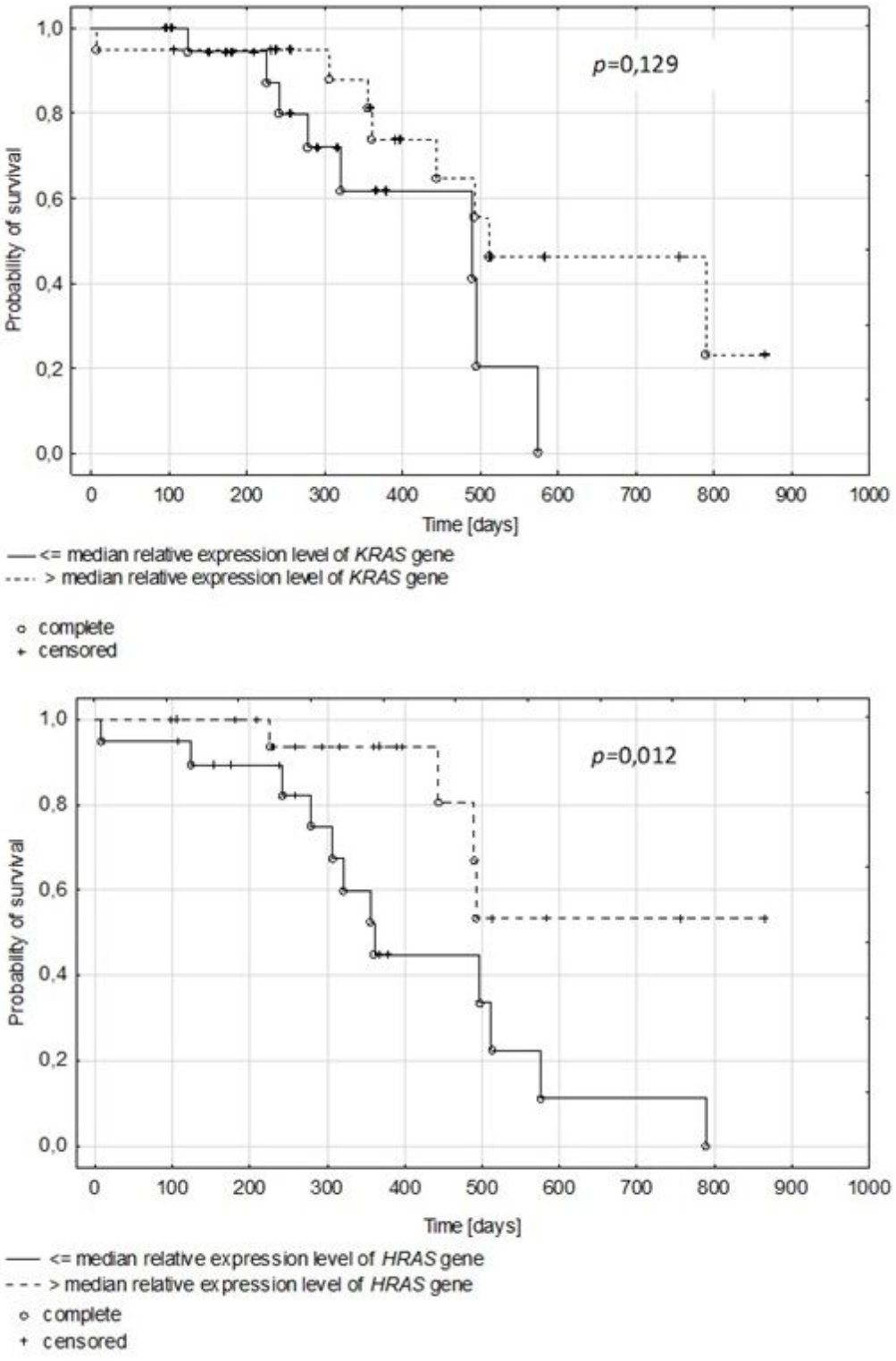
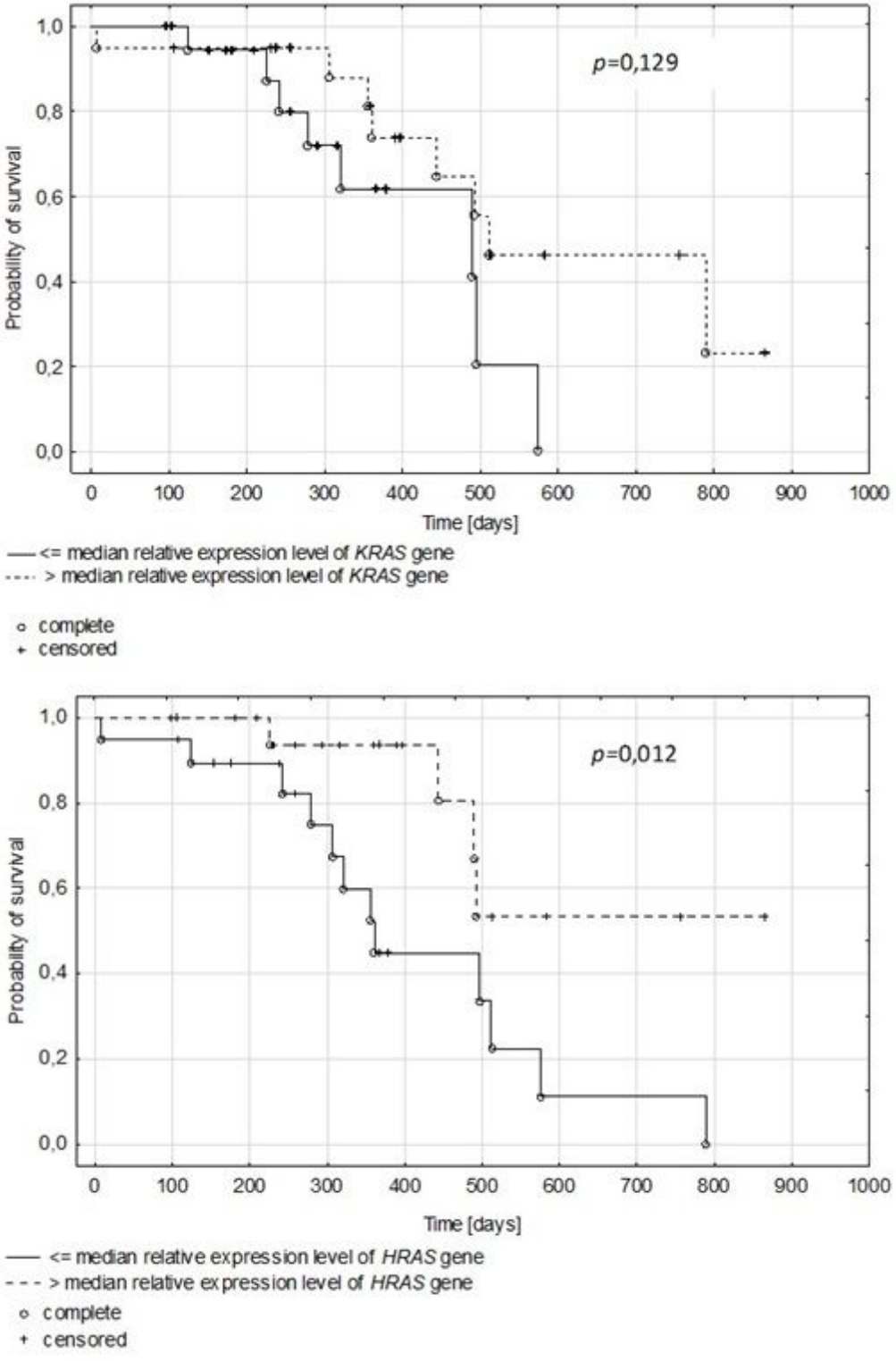


Figure 2

Kaplan-Meier survival curve for overall survival rates of patients with NSCLC based on the levels of relative KRAS (A) and HRAS (B) mRNA expression. Differences in survival between the low and high relative KRAS and HRAS expression groups were analysed using the log-rank test. The upper black dotted lines represent the high-expression group, whereas the lower line represents the low-expression group.



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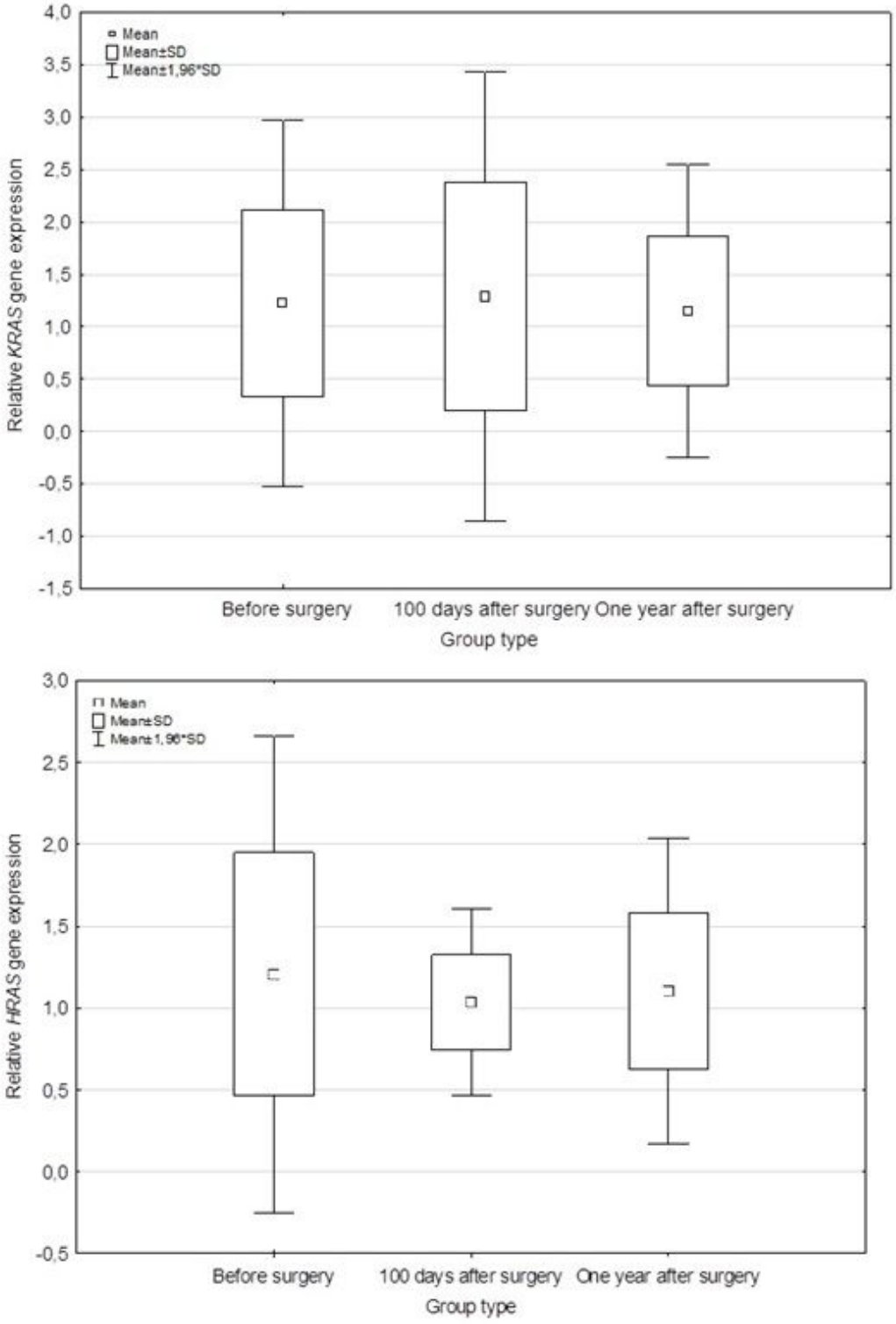
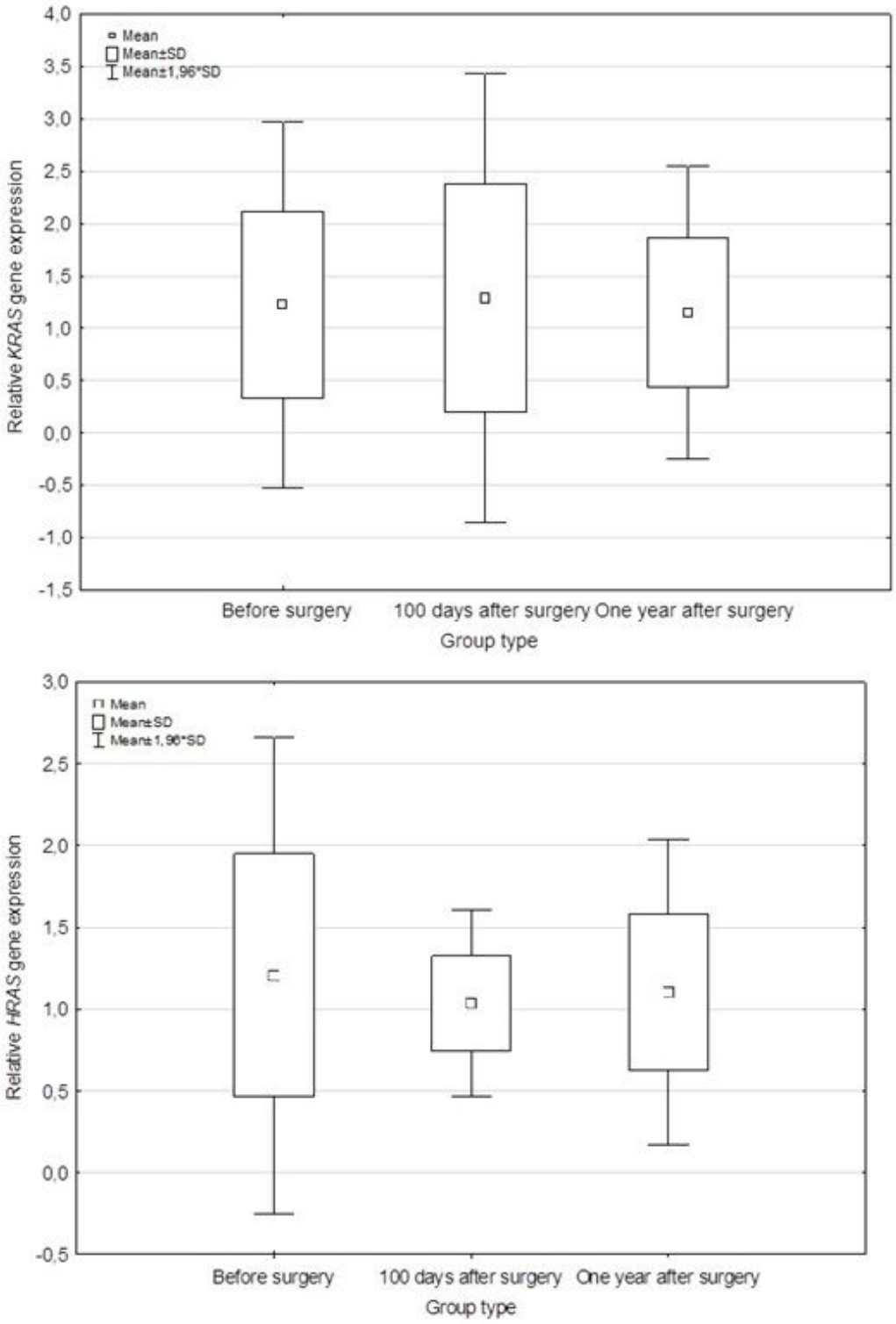


Figure 3

Relative KRAS and HRAS genes expression levels in non-small-cell lung cancer cases during the observation. Figure A shows the dependence of the relative KRAS gene expression levels and Figure B illustrates the relative HRAS gene expression levels based on time in which the whole blood samples were collected: before the surgery, 100 days after the surgery and one year after the surgery. The data plots for each of study groups represent the mean value ± the standard deviation.



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