



Musashi 2 (MSI2) expression as an independent prognostic biomarker in non-small cell lung cancer (NSCLC)

Iuliia Topchu^{1,2##}, Nikolai Karnaukhov^{3#}, Alexandra Mazitova^{1,2,###}, Veronika Yugai², Mark Voloshin³, Mariya Tikhomirova², Oleg Kit³, Elena Frantsiyants³, Leonid Kharin^{1,3}, Tamara Airapetova³, Ekaterina Ratner⁴, Alexey Sabirov⁴, Zinaida Abramova², Iliya Serebriiskii^{1,2}, Yanis Boumber^{1,2,5*}, Alexander Deneka^{1,2}

¹Molecular Therapeutics Program, Fox Chase Cancer Center, Philadelphia, PA, USA; ²Kazan Federal University, Kazan, Russian Federation; ³National Medical Research Center of Oncology, Rostov-on-Don, Russia, Russian Federation; ⁴Tatarstan Regional Clinical Cancer Center, Kazan, Russian Federation; ⁵Department of Hematology/Oncology, Fox Chase Cancer Center, Philadelphia, PA, USA

Contributions: (I) Conception and design: A Deneka, Y Boumber, Z Abramova, E Frantsiyants, T Airapetova; (II) Administrative support: O Kit, I Serebriiskii, Z Abramova; (III) Provision of study materials or patients: O Kit, E Frantsiyants, T Airapetova, E Ratner, A Sabirov; (IV) Collection and assembly of data: I Topchu, A Mazitova, N Karnaukhov, M Tikhomirova, M Voloshin, E Ratner, L Kharin; (V) Data analysis and interpretation: I Topchu, A Topchu, N Karnaukhov, M Tikhomirova, V Yugai; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Alexander Deneka. Molecular Therapeutics Program, Fox Chase, Cancer Center, Philadelphia, PA, 19111, USA.

Email: alexander.deneka@fccc.edu.

Background: Musashi-2 (MSI2) is a member of RNA-binding protein family that regulates mRNA translation of numerous intracellular targets and influences maintenance of stem cell identity. This study assessed MSI2 as a potential clinical biomarker in non-small cell lung cancer (NSCLC).

Methods: The current study included 40 patients with NSCLC, of whom one presented with stage I, 14 presented with stage II, 15 presented with stage III, and 10 patients had stage IV. All patients received standard of care treatments. All patient samples were obtained before treatment started. We used immunohistochemical (IHC) approach to measure MSI2 protein expression in matching specimens of normal lung versus tumor tissues, and primary versus metastatic tumors, followed by correlative analysis in relation to clinical outcomes. In parallel, clinical correlative analysis of MSI2 mRNA expression was performed *in silico* using publicly available datasets (TCGA/ICGC and KM plots).

Results: MSI2 protein expression in patient samples was significantly elevated in NSCLC primary tumors versus normal lung tissue (P=0.03). MSI2 elevated expression positively correlated with a decreased progression free survival (PFS) (P=0.026) combined for all stages and with overall survival (OS) at stage IV (P=0.013). Elevated MSI2 expression on RNA level was confirmed in primary tumor versus normal tissue samples in TCGA dataset (P<0.0001), and positively correlated with decreased OS (P=0.02). No correlation was observed between MSI2 expression and age, sex, smoking, and treatment type.

Conclusions: Elevated MSI2 expression in primary NSCLC tumors is associated with poor prognosis and can be used as a novel potential prognostic biomarker in NSCLC patients. Future studies in an extended patient cohort are warranted.

Keywords: Non-small cell lung cancer (NSCLC); overall survival (OS); RNA binding proteins; Musashi-2 (MSI2); prediction biomarkers

*Current address: Robert H Lurie Comprehensive Cancer Center and Division of Hematology/Oncology at the Department of Medicine, Feinberg School of Medicine, Northwestern University, Chicago, IL, 60611, USA.

**Current address: Department of Medicine, Cedars-Sinai Medical Center, Los Angeles, CA, 90048, USA.

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Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer-related mortality in the world, and metastasis is the most common cause of death in lung cancer patients (1). Despite the recent increase in the number of patients with this disease detected in the earlier stage that can often be treated successfully, they typically have poor prognosis, especially if tumors have begun to disseminate from the original site with a presence of occult metastasis (2). Therefore, it is imperative for the cancer community to develop not only drugs and other treatments effective in advanced stages as well as better adjuvant therapies, but also to search for the novel prognostic and predictive biomarkers in NSCLC.

The evolutionarily conserved translational regulator Musashi-2 (MSI2) is a member of Musashi family of RNA-binding proteins. It regulates mRNA translation of numerous intracellular targets, and influences multiple biological processes, including maintenance of stem cell identity (3). During embryogenesis, MSI2 plays a key role in regulation of asymmetric cell division *in Drosophila*, and proliferation, and differentiation in the nervous and hematopoietic tissues in mammals (4). MSI2 oncogenic properties were first demonstrated in a murine model of chronic myeloid leukemia in 2010 (5). Musashi proteins overexpression and prognostic significance were demonstrated in several cancer settings, including, but not limited to, colorectal and pancreatic cancers, glioblastomas, and several leukemias (6). Increased expression of MSI2 induced *in vitro* resistance to paclitaxel in ovarian cancer cells (7). Recently, the role for MSI2 in cancer was redemonstrated in regulation of aggressive breast cancer progression (8) and in supporting transformation of intraepithelial pancreatic neoplasia (PanIN) into ductal pancreatic adenocarcinoma (9).

Previously we reported that MSI2 is present in NSCLC tumors spontaneously arising in transgenic Kras/p53 murine model, upregulated in the metastasis-competent NSCLC murine and human cell lines, overexpressed in primary NSCLC versus normal lung, and progressively elevated in lung cancer patient samples (10). While we observed no correlation with survival in 123 patient NSCLC tissue microarray that mostly consisted of stage I and II patients,

we did observe that high levels of MSI2 mRNA are associated with inferior survival (hazard ratio 1.33) in a large NSCLC primary lung cancer samples database (10). In the current study, we performed additional comprehensive correlative analysis between MSI2 expression and clinical follow-up data, using an independent cohort of human NSCLC tissue samples, and different publicly available datasets to determine whether the degree of MSI2 expression varies between clinical-pathological profiles and whether it could be utilized as a novel marker of disease progression. We present the following article in accordance with the REMARK reporting checklist (available at <http://dx.doi.org/10.21037/jtd-20-2787>).

Methods

Patients

In the current retrospective study, we used randomly selected formalin fixed paraffin embedded (FFPE) NSCLC (n=40) and matching normal tissue specimens (n=20) collected from the Rostov National Medical Research Center Human Tissue Repository Facility (HTRF) in Rostov-on-Don, Russia, and the Republican Oncological Clinical Hospital's Repository, Kazan, Russia. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the local ethics committees of both organizations. Patients underwent a complete oncological resection and histological verification of tumor at both institutions and provided Institutional Review Board (IRB)—approved informed consents for storing tissue and reviewing of deidentified clinical data at the time of tissue acquisition (IRB No. 8, 02/13/2018 of the local ethics committee of the Kazan Federal University (Kazan, Russia) and IRB No.: 0.1.1.55-16/23/18-8 of National Medical Research Center of Oncology (Rostov-on-Don, Russia). Clinical information (Table 1) from the repository databases was abstracted in an anonymized fashion, and all samples were de-identified.

Immunohistochemistry of human NSCLC samples

Hematoxylin and eosin (H&E) stained sections were used

Table 1 Detailed clinical characteristics of NSCLC specimens

Characteristics	N
Gender	
Male	28
Female	12
Age at diagnosis, years	
Mean	55.5
Min	42
Max	75
SD	8.3
Histology	
Adenocarcinoma, NOS (8140/3)	27
Squamous cell carcinoma, NOS (8070/3)	8
Papillary adenocarcinoma, NOS (8260/3)	1
Adenosquamous carcinoma (8560/3)	2
Bronchiolo-alveolar adenocarcinoma, NOS (8250/3)	1
Non-small cell carcinoma (8046/3)	1
Overall stage	
I	1
IIA	12
IIB	2
IIIA	9
IIIB	5
IIIC	1
IVA	5
IVB	5
T stage	
1	4
2	25
3	3
4	7
x	1
N stage	
0	16
1	8
2	7

Table 1 (continued)**Table 1** (continued)

Characteristics	N
3	6
x	3
M stage	
0	33
1	6
x	1
Smoking status	
Smoker	25
Non-smoker	15
Treatment at stages I–III	
Chemotherapy and targeted therapy	3
Chemotherapy	15
Surgery	16
IV Stage treatment	
Chemotherapy	1
Chemotherapy/surgery and targeted therapy	6
Salvation surgery	3
IV Stage targeted therapy	
Anti-EGFR	2
Anti-Alk	1
Anti-VEGFR	3
Samples collection	
Bronchoscopy	19
Surgery	19
VATS	2

for morphological evaluation purposes and unstained sections were utilized for immunohistochemical (IHC) staining using standard methods. Briefly, 3–5 µm formalin-fixed, paraffin embedded sections were deparaffinized in 100% xylene for 3 times for 5 min each. Next, all slides were dehydrated in a sequence of ethanol (100%, 70%, 50%) solutions for 3 minutes each. Sections were then subjected to heat-induced epitope retrieval with 0.01 M citrate buffer (pH 6.0).

IHC staining was performed on Ventana Benchmark GX IHC/ISH system (Roche, Basel, Switzerland), using

primary MSI2 antibody (Abcam ab76148, Boston, MA, USA) and Mouse and Rabbit Specific HRP/DAB IHC Detection kit–Micro-polymer (Abcam ab236466, Boston, MA, USA) as a detection system. As a negative control, the primary antibody was replaced with normal mouse/rabbit IgG to confirm absence of specific staining. Additionally, all sections were stained using hematoxylin-eosin stain. In the end, all sections were dehydrated by immersion to a sequence of ethanol solutions (70%, 96%, 100%) and xylene for 30 seconds respectively. Coverslips were mounted using Histomount histological mounting medium (HS-103, National Diagnostics, Atlanta, GA, USA).

All slides were viewed on Leica Aperio CS2 scanner (Leica Biosystems, San Diego, CA, USA). MSI2 expression level in tissue was evaluated quantitatively using H-score approach. H-score was calculated as follows: the percentage of cells at each staining intensity level was calculated, and an H-score was assigned and calculated for each slide using the following formula: $[1 \times (\% \text{ cells } 1+) + 2 \times (\% \text{ cells } 2+) + 3 \times (\% \text{ cells } 3+)]$ (11). H-score values in the normal tissue ranged from 35 to 77. Median value of H-score of 170 was used as a cutoff for high MSI2 expression. The reason for this cutoff is because values above 170 represent top two quartiles of MSI2 expression H-scores. H-score quantitation was performed using QuPath digital pathology software (QuPath: Open Source Software for Digital Pathology Image Analysis, <https://qupath.github.io/>), as previously described (12).

TCGA analysis

For The Cancer Genome Atlas (TCGA) analysis, the most recent datasets for NSCLC (as of April, 2020) were accessed and analyzed using tools available at cBioPortal for Cancer Genomics (<http://www.cbioportal.org/>) (13). Kaplan-Meier analysis was performed using the KMPLLOT tool with automatically determined cutoff function, comparing high and low MSI2 mRNA expression in TCGA samples (<http://kmplot.com>) (14).

Statistical analysis

For statistical analysis of MSI2 expression (represented as H-score) we used Wilcoxon rank-sum test. The difference was determined as statistically significant in all cases with a P value <0.05 . The results were calculated using Microsoft Excel and GraphPad Prism software. The Kaplan-Meier method was used to construct survival curves, as well as to

calculate median survival. Difference between the groups was compared using a log-rank test. The correlation coefficients between protein expression and clinical and pathological parameters were estimated using Spearman correlation. Correlation analysis of MSI2 H-scores and clinical and pathological parameters correlations were performed using SPSS 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA). All p values are two-sided, and $P < 0.05$ is determined as statistically significant difference.

Results

NSCLC patient cohort and clinical characteristics

The cohort examined in the current study consists of 40 patients (70% male; 30% female). All patients signed a written consent for the study and processing of tissue samples in an anonymized fashion. The median age was 55 years (mean is 66 years, standard deviation 8.5 years; range, 38 to 75 years). One patient presented with stage I (2.5%), 14 and 15 patients presented with stage II and III (35% and 37.5%, respectively), and 10 patients (25%) had stage IV. All patients received treatment in accordance with the recommendations of the NCCN and did not participate in clinical trials (*Table 1*). All patient samples were obtained prior to standard of care treatment. Clinical (age, sex, smoking status) and histopathological (disease stage) characteristics (*Table 1*) had no impact on the patient outcome in terms of overall survival (OS) and progression-free survival (PFS) (P value >0.05) (*Table 2*). Overall, the cohort represented general population of NSCLC patients.

MSI2 levels are elevated in primary lung tumor tissue versus matching normal lung parenchyma

Based on analysis of lung cancer data in cBioportal database, we performed comparative analysis of MSI2 mRNA expression in a set of normal tissue (n=59) and lung adenocarcinoma samples (n=515) (*Figure 1A*). The analysis demonstrated, that MSI2 mRNA levels are statistically significantly elevated in primary tumor samples ($P < 0.0001$) compared to normal lung parenchyma. Next, we benchmarked this result to our dataset to reveal, if elevated MSI2 levels in primary lung adenocarcinoma tumors is a feature, represented solely on mRNA level, or it is observed on the protein level as well. Using our patient dataset, we utilized IHC approach to measure expression of MSI2 at the protein level (represented as H-score) in the normal

Table 2 Univariate analysis (log rank test) of patients' clinical characteristics influencing the overall survival (OS) and progression free survival (PFS)

Variable	N (%)	OS			PFS		
		Mean (months, 95% CI)	Log-rank	P value	Mean (months, 95% CI)	Log-rank	P value
Age (years)			0.636	0.425		2.131	0.144
< Median	19 (47.5%)	16.0 (13.8–18.2)			7.0 (6.0–8.0)		
> Median	21 (52.5%)	22.0 (13.2–30.8)			9.0 (8.1–9.9)		
SD	8.5						
Sex			0.051	0.822		0.528	0.467
Male	28 (70%)	15.0 (6.4–23.6)			7.0 (3.9–10.1)		
Female	12 (30%)	18.0 (15.8–20.2)			8.0 (6.3–9.7)		
Overall stage			3.619	0.164		1.037	0.596
II	15 (37.5%)	19.0 (13.9–24.1)			9.0 (7.8–10.2)		
III	15 (37.5%)	19.0 (15.3–22.7)			7.0 (5.2–8.8)		
IV	10 (25%)	12.0 (3.3–20.7)			4.0 (2.5–5.5)		
Total	40 (100%)						

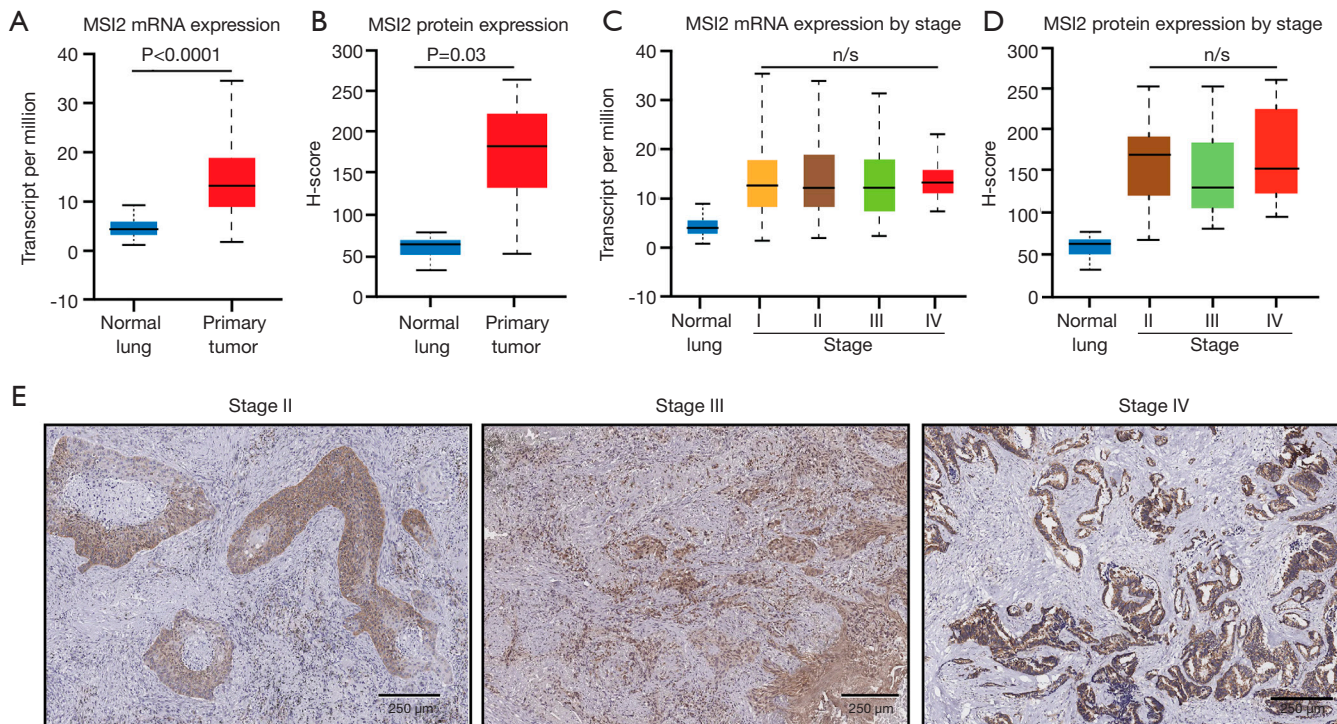


Figure 1 MSI2 levels in matching pairs of normal lung tissue versus NSCLC primary tumors. (A) MSI2 mRNA levels (RNA-seq data, obtained at cBioportal-TCGA) in 59 normal *vs.* 515 tumor tissue samples. (B) MSI2 H-scores, quantified by IHC, in 20 normal *vs.* 40 tumor patient samples. (C) MSI2 mRNA (RNA-seq) levels expression by stage in normal and tumor tissue samples. (D,E) MSI2 H-scores (D) and representative histopathological images (E) segregated by stage. Scale bar: 250 μ m.

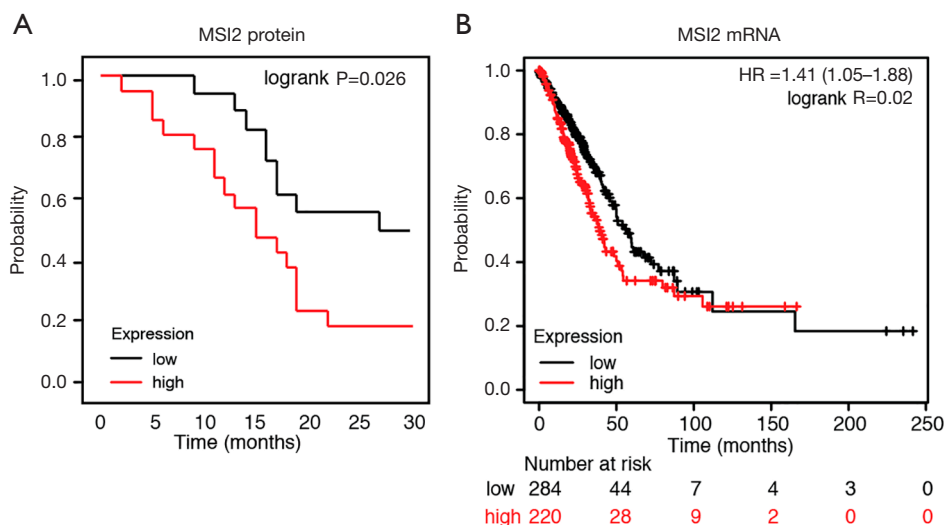


Figure 2 Elevated Musashi-2 levels in primary NSCLC correlate with decreased overall survival. (A) Kaplan-Meier analysis of patient overall survival with low and high levels of MSI2 protein (median H-score 170), as quantified via IHC approach in 40 samples. (B) Overall survival Kaplan-Meier analysis of TCGA RNA-seq data, MSI2 mRNA expression automatically split by optimal cutoff value (low vs. high) in 504 samples.

(n=20) and primary tumor (n=40) samples. This allowed us to determine, that MSI2 levels are elevated in primary tumor tissue (median H-score of 170), compared to normal parenchyma as well (P=0.03) (Figure 1B), and therefore we concluded that elevated levels of MSI2 in primary lung adenocarcinoma tumors is a constant feature, both on mRNA and protein levels.

In a subsequent analysis, aiming to determine if MSI2 levels are differentially altered with disease progression, we sorted out samples used in above analysis by disease stage and performed comparative analysis in a similar fashion in between stages. We did not observe statistically significant differences between MSI2 mRNA (Figure 1C) and protein expression (Figure 1D,E) levels depending of disease stage.

Elevated MSI2 levels in primary NSCLC tumors correlate with decreased PFS and OS

IHC approach is one of the most routinely used pathological diagnostics methods, and it can yield substantial data for further patient survival prediction. Kaplan-Meier and correlation analysis of MSI2 expression levels (as determined by IHC) and patient survival in our sample cohort demonstrated, that elevated MSI2 expression (H-score above 170) statistically significantly positively correlated with a decreased

PFS combined for all stages (P=0.026). Notably, high levels of MSI2 protein correlated with OS as well, but without statistical difference (P=0.065) (Figure 2A). This assumption is indirectly confirmed by further Kaplan-Meier analysis of MSI2 RNA levels and patients survival using publicly available datasets at cBioportal (14). Results demonstrate, that increased RNA levels of MSI2 in the primary lung tumors positively correlate with a decrease in OS (P=0.02) (Figure 2B), as revealed by survival analysis of TCGA RNA-seq data in NSCLC dataset using Pan-cancer RNA-seq KM plot tool (15). For the other clinical parameters, such as age, gender, smoking, and type of treatment there was no statistically significant correlation for MSI2 expression, both in our cohort of patients and TCGA dataset.

Stage IV NSCLC patients with low MSI2 levels have superior PFS and OS

Next, our goal was to determine, how levels of MSI2 in primary tumors as measured by IHC correlate with NSCLC patients survival during disease progression Univariate log-rank test relative analysis between MSI2 H-score levels and disease stage in our cohort demonstrated, that there were no statistically significant correlations between increased MSI2 levels on stages II and III and progression-

Table 3 Univariate analysis (log rank test) of MSI2 expression at different disease stage influencing the overall survival (OS) and progression free survival (PFS) the overall survival (OS) and progression free survival (PFS)

Variable	OS (months)			PFS (months)		
	Mean (95% CI)	Log-rank	P value	Mean (95% CI)	Log-rank	P value
Musashi-2 stage II		0.455	0.500		0.021	0.884
Low	27.0 (1.3–52.6)			8.0 (4.8–11.2)		
High	19.0 (17.7–20.3)			9.0 (5.1–12.9)		
Musashi-2 stage III		1.995	0.162		0.007	0.934
Low	24.0 (18.0–30.0)			7.0 (6.0–8.0)		
High	15.0 (6.7–23.3)			8.0 (6.8–9.2)		
Musashi-2 stage IV		6.223	0.013		4.116	0.042
Low	23.0 (15.5–30.5)			11.0 (0.0–26.3)		
High	6.0 (3.8–8.2)			2.0 (0.0–4.1)		
Musashi-2-all stages		3.393	0.065		4.986	0.026
Low	27.0 (21.9–32.1)			27.0 (19.7–34.3)		
High	17.0 (11.7–22.3)			15.0 (9.0–21.0)		

free or OS. However, analysis of stage IV samples revealed, that elevated levels of MSI2 protein (H-scores above median value of 170) statistically significantly correlated with decrease in PFS (P=0.042) and OS (P=0.013) (Table 3, Figure 3A), suggesting that NSCLC patients with low MSI2 levels at a later stage may have better survival.

Aiming to determine if the correlation differences (survival and MSI2 protein on stage IV *vs.* stages II and III) reflect elevated transcription of *MSI2* gene or depict enhanced protein activity and/or accumulation, we utilized KM plot tool to examine OS based on high and low levels of mRNA expression in TCGA NSCLC RNA-seq dataset. The limitation of TCGA dataset is that it had less than ten stage 4 NSCLC samples, so we were not able to directly compare our results. TCGA dataset analysis of stage I, II and III NSCLC revealed no statistically significant difference in survival between high and low MSI2 mRNA levels among these stages (Figure 3B), with borderline significance (P=0.042) at stage III.

Discussion

The RNA-binding protein Musashi-2 (MSI2) controls translation of proteins that support stem cell identity and lineage determination (6). Elevated expression of MSI2 plays an important role as a driver of tumor progression (16),

dissemination (9), and drug resistance (17,18), in numerous solid (8,19) and hematological malignancies (20–22). In lung cancer settings, previous publications demonstrate that MSI2 expression is elevated from an early stage NSCLC and suggest that MSI2 may play an important role in the reprogramming of transforming growth factor- β (TGF- β) signaling from growth-inhibiting to invasion-promoting during oncogenesis (10).

Despite significant advances in immune-oncology and targeted therapy in the recent years, durable responses in advanced lung cancer only occur in a fraction of patients. Therefore, one of the current challenges is to identify reliable biomarkers to predict therapeutic responses in this patient population. In the current work we for the first time assessed a novel stem cell marker MSI2 as a prediction marker of NSCLC progression. To confirm this assumption, it was necessary to conduct studies not only in the cell lines of NSCLC, but also to perform correlation and IHC analyses of tumor and normal tissue samples obtained from patients with NSCLC.

Our results confirm previously published data on elevated MSI2 levels in primary NSCLC tumors regardless of the stage (10) in an independent patient samples cohort, and for the first time demonstrate that high MSI2 expression levels in primary NSCLC tumors correlate with poor patients survival and prognosis, censored based on three year

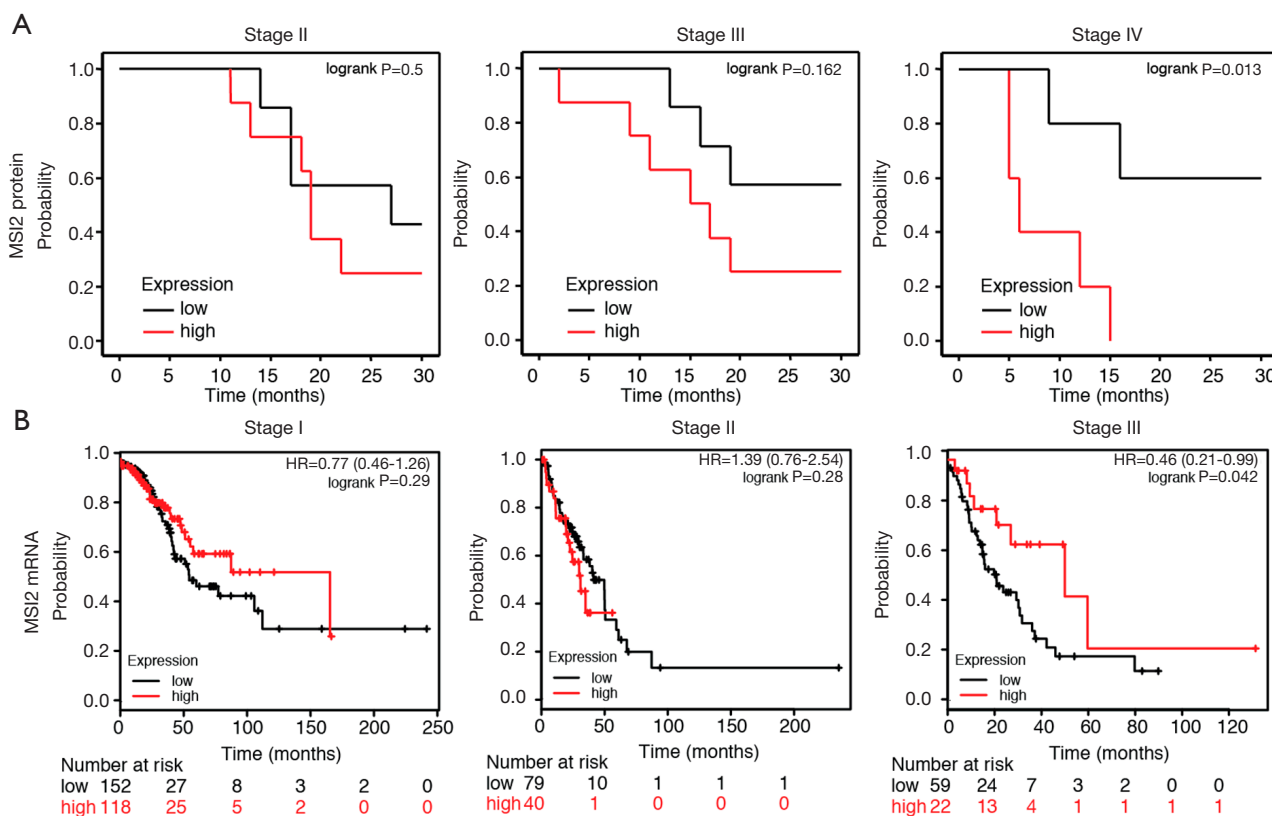


Figure 3 NSCLC patients with low MSI2 levels have better overall survival. (A) Kaplan-Meier analysis of patient overall survival with low and high levels of MSI2 protein (median H-score 170), as quantified via IHC approach, segregated by stage (n=39, stages 2–4). (B) Kaplan-Meier analysis of overall survival using TCGA MSI2 mRNA expression dataset with automatically determined optimal cutoff value (low vs. high), divided by stage (total n=470, stages 1–3).

survival. We observed a statistically significant correlation between high MSI2 expression and lower PFS, but not with OS. This is probably due to relatively small cohort size and can potentially improve and yield statistically more robust values with an increase in number of the samples.

These data can potentially nominate MSI2 to be used as a novel prognostic biomarker in patients with NSCLC. Elevated MSI2 levels in tumor versus normal tissues were detected in extended RNA-seq TCGA dataset, and confirmed in our patients dataset by IHC staining, with comparable results presented. Gene expression profiling by RNA sequencing is now commonly used to profile and classify the cell types present in a tissue, discover new gene signatures for a cell type or pathway, elucidate the developmental lineage of a cell type, and compare different biological states or conditions (23). One advantage of this study is that we used IHC approach to validate RNA-seq

findings of gene expression and to evaluate expression of MSI2 in patient samples. Considering the heterogeneity of the tumor, IHC approach allows to evaluate larger area of the tumor and reduce errors in the qualitative and quantitative assessment of expression of the marker of interest, and is one of the most common methods utilized in clinical practice, that can be used to validate the findings from a broad, hypothesis-free approach such as RNA-seq to draw accurate conclusions (24). This, on one hand, can explain demonstrated certain level of discrepancy in results of correlative analysis performed for samples with MSI2 levels measured at protein (via IHC) and mRNA (via RNA-seq) levels. On the other hand, this may reflect underlying intrinsic biological differences, depending on the underlying mechanism of disease progression, when the tumors potentially become more dependent on MSI2 protein activity at later stages of NSCLC oncogenesis.

Conclusions

Based on the clinical and statistical data obtained, we can state a significant role for MSI2 in the processes of carcinogenesis and the progression of the NSCLC. High expression of MSI2 in late-stage tumors and its correlation with decrease in patient survival suggest a higher aggressiveness of the disease in this subset of patients. Measuring MSI2 protein expression in primary NSCLC tumors can be used as a novel potential prognostic biomarker in NSCLC patients. Future studies evaluating MSI2 in an extended patient cohort are warranted.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki

(as revised in 2013). The study was approved by joint institutional ethics board of the Republican Oncological Clinical Hospital, Kazan, Russia and Rostov National Medical Research Center, Rostov-on-Don, Russia (No. 8, 02/13/2018 and No.: 0.1.1.55-16/23/18-8 and). Informed consent was taken from all individual participants.

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