

## Prognostic significance of miR-23b in combination with P-gp, MRP and LRP/MVP expression in non-small cell lung cancer

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Recently, miR-23b has emerged as a promising new cancer biomarker but its role in lung cancer has not been established yet. Patients still do not respond well to available treatments, probably due to expression of multidrug resistance (MDR) proteins, such as P-gp, MRP and LRP/MVP. The aim of this study was to determine the role of miR-23b in non-small cell lung cancer (NSCLC) and its relationship to the patient outcome together with MDR transporter proteins. We immunohistochemically evaluated expression of P-gp, MRP and LRP/MVP and quantified the relative levels of miR-23b in 62 NSCLC patients' samples. The prognostic significance of miR-23b and MDR proteins was tested by Kaplan-Meier and Cox-regression analysis. Our results showed that miR-23b is mostly downregulated in NSCLC samples (57/62) and that its upregulation in tumors is connected with longer progression-free survival (PFS;  $P = 0.065$ ) and overall survival (OS;  $P = 0.048$ ). The Cox proportional hazard model revealed that the risk of death or relapse in NSCLC patients with miR-23b downregulation increases together with LRP/MVP expression and both risks decrease with miR-23b upregulation ( $HR_{PFS} = 4.342$ ,  $P_{PFS} = 0.022$ ;  $HR_{OS} = 4.408$ ,  $P_{OS} = 0.015$ ). Our findings indicate that miR-23b, especially in combination with LRP/MVP expression, might serve as a suitable prognostic biomarker for NSCLC patients.

*Key words: non-small cell lung cancer (NSCLC), MiR-23b, P-gp, MRP, LRP/MVP, prognosis*

Lung cancer is a major cause of cancer mortality worldwide and non-small cell lung cancer (NSCLC) represents approximately 80-85% of all cases [1]. The problem is that many NSCLC patients do not respond to the therapy due to the emergence of multidrug resistance (MDR). The most studied proteins underlying this phenomenon are P-glycoprotein (P-gp/ABCB1/MDR1), Multidrug resistance-related protein 1 (MRP/ABCC1/MRP1) and Lung-resistance related protein/Major vault protein (LRP/MVP).

P-gp and MRP belong to the family of ATP binding cassette (ABC) transporters. P-gp actively transports hydrophobic, positively charged and neutral molecules, while MRP pumps negatively charged molecules (mainly glutathione conjugates) from the cell to the extracellular space [2-5]. They are localized in the cytoplasmic membrane and they are normally expressed in organs, such as lung, intestine or the hemato-encephalic barrier which are in contact with environment and xenobiotics (toxins and drugs). The physiological function of P-gp and

MRP is to export toxic metabolites and xenobiotics entering the cells from the environment and to protect the cells from damage [6-10]. These ABC-transporters are aberrantly expressed in tumor cells. Activation of their expression in cancer cells is responsible for the export of diverse chemotherapeutics from the cells [5, 7, 8, 11, 12].

LRP/MVP is a ribonucleoprotein particle able to transport drugs such as platinum derivatives from the nucleus to the cytoplasm. After that, these compounds can be transported outside from the cells by ABC-transporters [13-15].

Expression of P-gp, MRP and LRP/MVP is often observed in NSCLC samples and it is believed that these molecules play a major role in the emergence of multidrug resistance [5, 7, 8, 16].

Recent studies have shown that miRNAs play an important role in development, differentiation, DNA damage response, proliferation, apoptosis and other processes. They are also involved in diverse regulatory pathways and they can affect

carcinogenesis, chemoresistance and radioresistance [17-21]. For these reasons, miRNAs whose expression may be increased or decreased in tumors could be called a class of oncogenes or tumor suppressors and they might be used as biomarkers for monitoring carcinogenesis [17, 22]. Radiotherapy, chemotherapy and biological therapy are frequently used for treating advanced forms of NSCLC but in recent years, RNA interference (RNAi) has also been introduced [1, 23]. RNAi is a highly conserved molecular mechanism of post-transcriptional gene silencing. Functional units of RNAi in humans are mainly small (~22 nucleotides) non-coding, endogenous, single-stranded RNAs, called microRNAs (miRNAs). Based on complementarity, miRNAs bind to the target mRNA which is either completely degraded or prevented from translation [23]. Currently, according to miRBase 21, 2588 mature human miRNAs are known to regulate many protein-coding genes [24].

Recently, miR-23b has emerged as a promising molecule that may be involved in tumor development and metastasis. It behaves as a tumor-suppressor or as an oncogenic miRNA in time- and tissue-dependent manner [25]. Owing to its dual role, miR-23b in tumors targets different molecules and regulates oncogenes supporting proliferation and metastasis formation, such as Src kinase, AKT, SNAIL and ZEB1, or tumor-suppressors having an apoptotic and anti-metastatic effect (for example Nischarin, VHL and PTEN) [26-31]. Downregulation of miR-23b was observed in prostate, bladder and thyroid cancer. Patients with higher levels of miR-23b had better prognosis than those with lower levels [26, 28, 32]. Upregulation of miR-23b was detected in renal, breast cancer and gliomas [29-31] and its upregulation was connected with poor patient's outcome.

Prognostic significance of miR-23b has not been studied in NSCLC yet. For this reason, we quantified level of miR-23b in NSCLC patient samples. Identification of its significance could be crucial for its usage as a biomarker. We compared its expression with the expression of known transporter proteins P-gp, MRP and LRP/MVP involved in the emergence of MDR. Finally, we created a regression model for assessing patient risk of death or relapse.

## Patients and methods

**Patients and their clinical data.** Formalin-fixed and paraffin-embedded (FFPE) tissue samples of lung cancer from 1996-2000 were obtained from the archives of the Department of Clinical and Molecular Pathology, Faculty of Medicine and Dentistry, Palacky University Olomouc and University Hospital Olomouc. The sample comprised 62 patients with lung cancer, 55 males and 7 females (age range 33 to 78 years), after approval by the ethics committee of University Hospital and Faculty of Medicine and Dentistry, Palacky University Olomouc. In this cohort, 30 tumors were classified as adenocarcinomas (ADCs), 26 as squamous cell carcinomas (SCCs) and 6 as large cell carcinomas (LCCs). 18 patients were in stage

I/II and 33 patients in stage III/IV. For the rest of the patients, the stage was unknown. 22 patients received chemotherapy. 17 of them underwent platinum based chemotherapy regime and the rest were treated with different chemotherapeutics, such as fluorouracil, doxorubicin or taxanes. The characteristics of patients are summarised in Table 1. The clinicopathological parameters, progression free survival (PFS) and overall survival (OS), were monitored over 175 months.

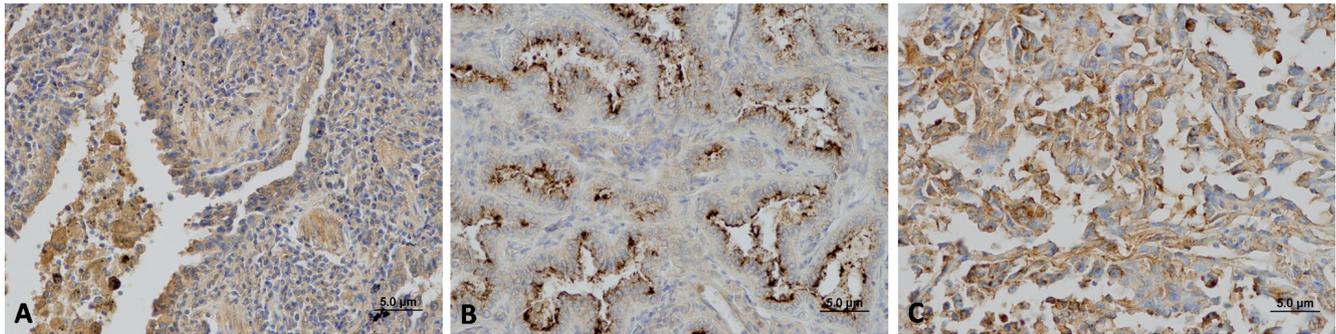
**Tissue microarray construction.** Tumor tissue microarrays (TMAs) were constructed using 62 formalin-fixed and paraffin-embedded primary lung cancer specimens. The tissue areas for sampling were selected by pathologists on the basis of visual alignment with corresponding H&E stained section and were taken from two to four tissue cores from a tumor block and were replaced into recipient paraffin block using a tissue microarrayer Galileo TMA CK3500 (BioRep, Milan, Italy).

**Immunohistochemical staining of P-gp, MRP and LRP/MVP.** The TMA sections were deparaffinized and an indirect immunohistochemistry technique was used. Then LRP/MVP antigen was unmasked in citrate buffer (pH 6) and MRP in Target Retrieval Solution, High pH (10x) (Dako, Glostrup, Denmark). The sections for detection of P-gp were not pre-treated because the ABCB1 / P Glycoprotein Mouse anti-Human Monoclonal (UIC2) Antibody (LS-C58240-100, LifeSpan Biosciences, Inc., Seattle, WA, USA) targets the extracellular epitope of P-gp protein [33]. This antibody was diluted in a ratio 1:200. Both Monoclonal Antibody to MRP1 (human) (MRPm5) (ALX-801-012-C125, Enzo Life Sciences, Inc., Farmingdale, NY, USA) and Monoclonal Antibody to MVP/LRP (human), mAb (MVP-37) (LMR5) (ALX-801-026-C125, Enzo Life Sciences, Inc., Farmingdale, NY, USA)

**Table 1. Characteristics of patients.**

| Characteristics      | Subgroups     | Number | %     |
|----------------------|---------------|--------|-------|
| Gender               | Male          | 55     | 88.7  |
|                      | Female        | 7      | 11.3  |
| Age in years         | < 60          | 31     | 50.0  |
|                      | ≥ 60          | 31     | 50.0  |
| Histological subtype | ADC           | 30     | 48.4  |
|                      | SCC           | 26     | 41.9  |
|                      | LCC           | 6      | 9.7   |
| Stage at diagnosis   | I             | 13     | 21.0  |
|                      | II            | 5      | 8.1   |
|                      | IIIA          | 20     | 32.3  |
|                      | IIIB          | 5      | 8.1   |
|                      | IV            | 8      | 12.9  |
|                      | Not specified | 11     | 17.7  |
| Chemotherapy         | Yes           | 22     | 35.5  |
|                      | No            | 31     | 50.0  |
|                      | Not specified | 9      | 14.5  |
| Total                |               | 62     | 100.0 |

ADC – adenocarcinoma, SCC – squamous cell carcinoma, LCC – large cell carcinoma.



**Figure 1.** Immunohistochemical expression of P-gp, MRP and LRP/MVP. Representative images of immunohistochemical staining of P-gp (A), MRP (B) and LRP/MVP (C) in NSCLC samples. Magnification 200x.

were used at a dilution 1:200. Visualisation was made by EnVision™+ Dual Link (Dako, Glostrup, Denmark) and nuclei were counterstained with haematoxylin. Stained sections were observed under an optical microscope and images were captured with a DP71 camera (Olympus, Tokyo, Japan). The expression of P-gp, MRP and LRP/MVP were semi quantitatively assessed by two pathologists by estimation of the percentage of positive cells as very low ( $\leq 0.1$ ), low ( $\leq 0.3$ ), moderate ( $\leq 0.6$ ) or high ( $> 0.6$ ).

**Isolation of total RNA.** Total RNA isolation from corresponding tissue cores obtained by tissue microarrayer Galileo TMA CK3500 [34] was performed using the RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The concentration of total RNA was measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) and then RNA was stored at  $-80^{\circ}\text{C}$  until use.

**Reverse transcription.** TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) was used according to manufacturer's protocol. For reverse transcription, 10 ng of total RNA was used and total volume of reaction was 15  $\mu\text{l}$ . Pooled gene-specific primers for miR-23b and RNA, U6 small nuclear 2 (RNU6B) (Applied Biosystems, Foster City, CA, USA) were added to the reverse transcription polymerase chain reaction (RT-PCR). RT-PCR product was then pre-amplified according to protocol using TaqMan® PreAmp Master Mix (2x) (Applied Biosystems, Foster City, CA, USA) with pooled TaqMan® MicroRNA Assays (Applied Biosystems, Foster City, CA, USA) for miR-23b and RNU6B.

**Real-time PCR for miR-23b quantification.** The pre-amplified PCR product was diluted in DEPC water (1:20). Each sample was analysed in triplicate and the volume per each reaction was 10  $\mu\text{l}$ . The reaction consisted of TaqMan® Universal PCR Master Mix (2x) (Applied Biosystems, Foster City, CA, USA), DEPC water, corresponding TaqMan® MicroRNA Assay and diluted pre-amplified PCR product. Real-time PCR was performed using LightCycler® 480 (Roche, Branford,

CT, USA) with appropriate software according to protocol and RNU6B was used as an endogenous control. The non-tumor control sample was prepared by pooling commercially available Human Lung Total RNAs (AM7968, Lot. 0904002 and 1203010, Applied Biosystems, Foster City, CA, USA) and Total RNAs, Lung, Human (540019, Lot. 0006051745 and 0006079356, Agilent Technologies, Santa Clara, CA, USA). The relative expression level of miR-23b was calculated using the following equation: relative gene expression =  $2^{-(\Delta\text{Ct}_{\text{sample}} - \Delta\text{Ct}_{\text{control}})}$ .

**Statistical analysis.** Statistical analysis was performed using software SPSS, version 20.0. Pearson's Chi-square was used to assess the relationships between categorical variables. Mann-Whitney test was used to evaluate difference between groups. Survival analysis was done by Kaplan-Meier method with the log-rank test applied for comparison of curves. Overall survival (OS) was defined as time from the surgery until patient's death, progression free survival (PFS) as time from surgery until disease relapse or patient's death. Cox proportional hazard model was created for assessing the risk of patient's death and/or relapse of disease.  $P < 0.05$  was considered as statistically significant.

## Results

TMA blocks from 62 patients were immunohistochemically analysed for P-gp, MRP and LRP/MVP expression. The tissues from TMAs were also used for the quantification of miR-23b expression.

**Expression of P-gp, MRP and LRP/MVP in NSCLC.** The expression of P-gp, MRP and LRP/MVP was assessed in 62 patients (Figures 1, 2). The P-gp was detected in 40 (64.5 %) patients, MRP in 50 (80.6 %) patients and expression of LRP/MVP was found in 35 (56.4 %) patients. Expression of these proteins in different histological subtypes is summarised in Table 2. There was no significant difference in P-gp, MRP or LRP/MVP expression between ADC, SCC and LCC specimens ( $P_{\text{P-gp}} = 0.313$ ,  $P_{\text{MRP}} = 0.432$ ,  $P_{\text{LRP/MVP}} = 0.108$ ) (Figure 3). For the next analysis, very low expression of proteins was considered

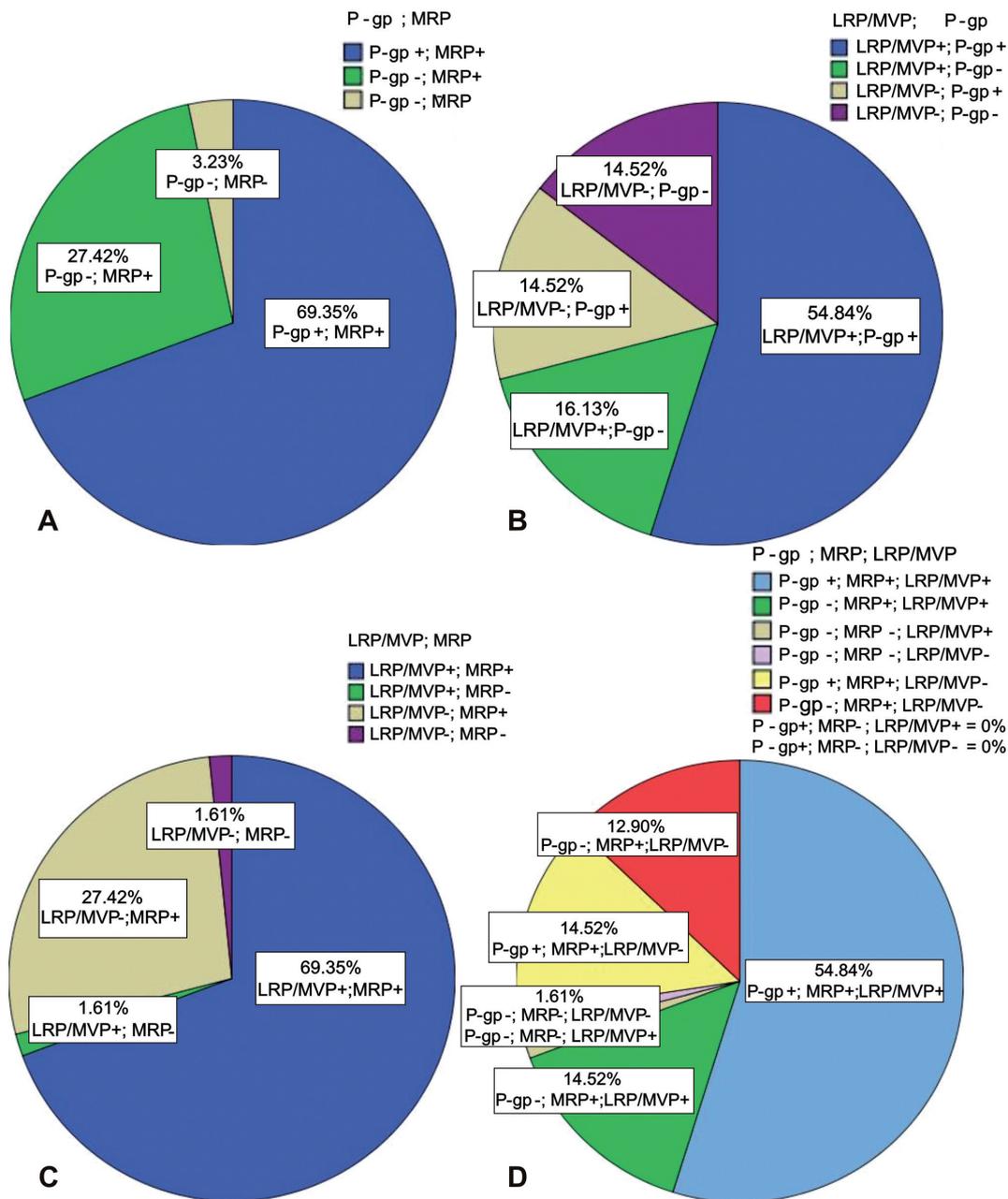


Figure 2. Combined expression of P-gp, MRP and LRP/MVP. Pie charts show percentage of combined expression of P-gp and MRP (A), LRP/MVP and P-gp (B), LRP/MVP and MRP (C) and all three analysed protein markers P-gp, MRP and LRP/MVP (D).

as a negative expression while low, moderate and high were considered as a positive expression.

**Expression of miR-23b in NSCLC.** The relative quantification of mature miR-23b in 62 lung cancer samples was performed. Higher expression of miR-23b compared to non-tumor control RNA was detected in 5 (8.1%) samples and lower expression in 57 (91.9 %) samples. There was no significant difference in miR-23b expression between specimens from different histological subtypes (ADC, SCC, LCC;

$P = 0.711$ ; Table 3). For the next analysis, higher expression of miR-23b ( $2^{-\Delta\Delta Ct} > 1$ ) was marked as “up” (means upregulation) and lower expression ( $2^{-\Delta\Delta Ct} < 1$ ) was marked as “down” (means downregulation).

**Relationship of miR-23b, P-gp, MRP and LRP/MVP expression with clinicopathological parameters.** Progression free survival and overall survival were used as the clinicopathological parameters for evaluating the prognostic role of these variables in NSCLC patients. Patient outcome was monitored

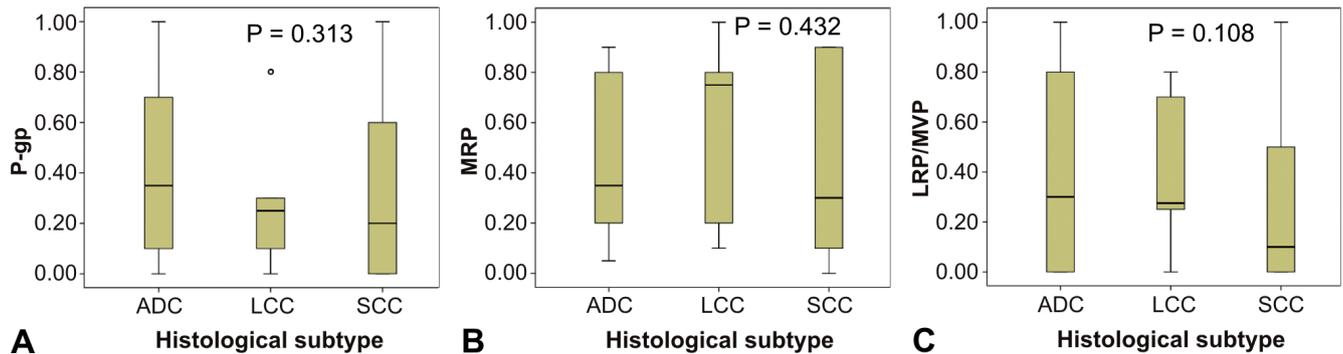


Figure 3. Analysis of P-gp, MRP and LRP/MVP expression in different histological subtypes. Box plots show the proportion of P-gp (A), MRP (B) and LRP/MVP (C) expression in different histological subtypes of NSCLC.

ADC – adenocarcinoma, SCC – squamous cell carcinoma, LCC – large cell carcinoma.

Table 2. Summary of expression of P-gp, MRP and LRP/MVP in different histological subtypes.

| Protein | Histological subtype | Intensity               |                    |                         |                  | Statistics       |       |
|---------|----------------------|-------------------------|--------------------|-------------------------|------------------|------------------|-------|
|         |                      | Very low ( $\leq 0.1$ ) | Low ( $\leq 0.3$ ) | Moderate ( $\leq 0.6$ ) | High ( $> 0.6$ ) | Pearson $\chi^2$ | P     |
| P-gp    | ADC                  | 8                       | 7                  | 3                       | 12               | 2.324            | 0.313 |
|         | SCC                  | 12                      | 6                  | 2                       | 6                |                  |       |
|         | LCC                  | 2                       | 3                  | 0                       | 1                |                  |       |
|         | Total [No. (%)]      | 22 (35.5)               | 16 (25.8)          | 5 (8.1)                 | 19 (30.6)        |                  |       |
| MRP     | ADC                  | 4                       | 11                 | 4                       | 11               | 1.679            | 0.432 |
|         | SCC                  | 7                       | 7                  | 1                       | 11               |                  |       |
|         | LCC                  | 1                       | 1                  | 0                       | 4                |                  |       |
|         | Total [No. (%)]      | 12 (19.4)               | 19 (30.6)          | 5 (8.1)                 | 26 (41.9)        |                  |       |
| LRP/MVP | ADC                  | 11                      | 4                  | 3                       | 12               | 4.457            | 0.108 |
|         | SCC                  | 15                      | 2                  | 5                       | 4                |                  |       |
|         | LCC                  | 1                       | 3                  | 0                       | 2                |                  |       |
|         | Total [No. (%)]      | 27 (43.5)               | 9 (14.5)           | 8 (12.9)                | 18 (29.0)        |                  |       |

Comparison of negative ( $\leq 0.1$ ) and positive ( $\geq 0.1$ ) expression of MDR proteins in different histological subtypes. MDR – multidrug resistance, P-gp – P-glycoprotein, MRP – Multidrug resistance-related protein 1, LRP/MVP – Lung-resistance related protein/Major vault protein, ADC – adenocarcinoma, SCC – squamous cell carcinoma, LCC – large cell carcinoma.

over 175 months. Individual data are showed in Table 4. Expression of P-gp, MRP and LRP/MVP in patients with regard to miR-23b expression levels is summarised in Table 5. The Kaplan-Meier analysis showed a trend to association between PFS ( $P = 0.065$ ) and statistically significant relationship be-

Table 3. Expression of miR-23b in different histological subtypes.

| Histological subtype | miR-23b |    | Total |
|----------------------|---------|----|-------|
|                      | down    | up |       |
| ADC                  | 27      | 3  | 30    |
| SCC                  | 24      | 2  | 26    |
| LCC                  | 6       | 0  | 6     |
| Total                | 57      | 5  | 62    |

Summary of miR-23b relative expression in different histological subtypes based on  $2^{-\Delta\Delta Ct}$  values.

Down – downregulation ( $2^{-\Delta\Delta Ct} < 1$ ), up – upregulation ( $2^{-\Delta\Delta Ct} > 1$ ).

tween OS ( $P = 0.048$ ) and miR-23b expression (Figure 4A, B). Survival analysis showed no significant association between expression of P-gp, MRP and LRP/MVP and patient's outcome (P-gp:  $P_{PFS} = 0.993$ ,  $P_{OS} = 0.982$ ; MRP:  $P_{PFS} = 0.911$ ,  $P_{OS} = 0.945$ ; LRP/MVP:  $P_{PFS} = 0.908$ ,  $P_{OS} = 0.966$ ; Figure 4C-H). Based on these results, overexpression of miR-23b seems to be good prognostic factor in the case of NSCLC patients.

**Cox regression hazard model.** The relationship between miR-23b and P-gp, MRP and LRP/MVP was first analysed by Chi-Square and Mann-Whitney tests. Neither of them showed any significant association between the variables (Table 6).

The Cox proportional hazard model was created to assess the relationship between the risk of death or relapse and variables of interest such as miR-23b and LRP/MVP. The association between the risk and the above variables were statistically significant (PFS:  $P = 0.022$ , HR = 4.342, CI (95%) = 1.231-15.318, Wald = 5.211; OS:  $P = 0.015$ , HR = 4.408, CI (95%) = 1.356-16.350, Wald = 5.949) and model fit parameters

**Table 4. Individual data on tumor classification and expression levels of analysed markers.**

| Patient No. | Gender | Histological subtype | Classification |   |   | P-gp | MRP | LRP/MVP | miR-23b |
|-------------|--------|----------------------|----------------|---|---|------|-----|---------|---------|
|             |        |                      | T              | N | M |      |     |         |         |
| 1           | M      | ADC                  |                |   |   | 0.0  | 0.9 | 0.0     | 0.00    |
| 3           | M      | SCC                  | 2              | 2 | 0 | 0.0  | 0.0 | 0.0     | 0.00    |
| 4           | M      | LCC                  | 2              | 0 | 0 | 0.1  | 0.8 | 0.1     | 0.17    |
| 5           | M      | ADC                  | 1              | 0 | 0 | 0.3  | 0.9 | 0.2     | 0.18    |
| 6           | M      | ADC                  | 2              | 0 | 0 | 0.0  | 0.9 | 0.7     | 0.09    |
| 7           | M      | ADC                  | 1              | 0 | 0 | 0.3  | 0.8 | 0.1     | 0.09    |
| 8           | F      | SCC                  | 3              | 2 | 0 | 0.6  | 0.3 | 0.9     | 0.56    |
| 9           | M      | SCC                  | 3              | 1 | 0 | 0.0  | 0.1 | 0.5     | 1.73    |
| 10          | M      | SCC                  |                |   |   | 0.3  | 0.9 | 0.0     | 0.00    |
| 11          | M      | SCC                  | 2              | 0 | 0 | 0.0  | 0.9 | 1.0     | 0.00    |
| 12          | M      | ADC                  | 2              | 0 | 0 | 0.0  | 0.6 | 0.0     | 0.04    |
| 13          | M      | SCC                  | 2              | 2 | 0 | 0.6  | 0.1 | 0.0     | 0.07    |
| 14          | M      | SCC                  | 1              | 0 | 0 | 0.0  | 0.9 | 0.0     | 0.01    |
| 15          | M      | ADC                  | 4              | 2 | 0 | 0.3  | 0.2 | 0.7     | 0.15    |
| 16          | M      | ADC                  | 2              | 2 | 0 | 1.0  | 0.3 | 1.0     | 0.06    |
| 17          | M      | ADC                  | 3              | 2 | 0 | 0.0  | 0.2 | 1.0     | 0.36    |
| 19          | M      | SCC                  | 3              | 1 | 0 | 0.0  | 0.3 | 0.0     | 0.15    |
| 20          | M      | SCC                  |                |   |   | 0.0  | 0.8 | 0.0     | 0.05    |
| 21          | M      | SCC                  | 2              | 0 | 0 | 0.0  | 0.8 | 0.4     | 0.04    |
| 22          | M      | SCC                  | 2              | 2 | 0 | 0.0  | 0.0 | 0.1     | 0.03    |
| 23          | M      | SCC                  |                |   |   | 0.0  | 0.9 | 0.0     | 0.11    |
| 24          | M      | ADC                  | 1              | 0 | 0 | 0.0  | 0.8 | 0.0     | 3.38    |
| 25          | F      | ADC                  | 4              | 2 | 1 | 0.8  | 0.1 | 0.2     | 0.09    |
| 26          | M      | ADC                  | 2              | 0 | 1 | 0.0  | 0.3 | 1.0     | 0.23    |
| 27          | M      | SCC                  | 4              | 0 | 0 | 0.0  | 0.1 | 0.5     | 0.14    |
| 28          | M      | ADC                  | 3              | 2 | 1 | 0.8  | 0.2 | 0.5     | 0.13    |
| 29          | M      | ADC                  |                |   |   | 0.2  | 0.8 | 0.0     | 0.15    |
| 30          | F      | ADC                  | 2              | 2 | 0 | 0.7  | 0.2 | 0.0     | 0.15    |
| 31          | M      | ADC                  |                |   |   | 1.0  | 0.8 | 0.9     | 0.17    |
| 32          | M      | ADC                  | 1              | 0 | 0 | 0.1  | 0.8 | 0.2     | 0.04    |
| 33          | M      | LCC                  | 2              | 1 | 0 | 0.2  | 1.0 | 0.3     | 0.10    |
| 34          | M      | SCC                  | 2              | 2 | 0 | 0.0  | 0.1 | 0.0     | 0.18    |
| 35          | M      | SCC                  | 2              | 1 | 0 | 0.3  | 0.9 | 0.0     | 0.26    |
| 36          | M      | SCC                  | 3              | 1 | 0 | 1.0  | 0.2 | 0.8     | 0.24    |
| 37          | M      | ADC                  | 2              | 2 | 0 | 0.3  | 0.2 | 0.9     | 0.03    |
| 38          | M      | SCC                  | 2              | 2 | 0 | 0.7  | 0.3 | 0.0     | 0.15    |
| 39          | M      | LCC                  | 1              | 2 | 0 | 0.3  | 0.1 | 0.8     | 0.49    |
| 40          | M      | LCC                  | 1              | 1 | 0 | 0.0  | 0.7 | 0.7     | 0.25    |
| 41          | M      | SCC                  | 2              | 2 | 0 | 0.0  | 0.2 | 0.1     | 0.05    |
| 42          | F      | ADC                  | 1              | 0 | 0 | 0.4  | 0.9 | 0.1     | 0.27    |
| 43          | M      | ADC                  | 4              | 1 | 0 | 0.9  | 0.4 | 0.8     | 21.95   |
| 44          | M      | ADC                  | 2              | 2 | 0 | 0.9  | 0.1 | 0.0     | 0.97    |
| 45          | F      | ADC                  |                |   |   | 0.1  | 0.9 | 0.4     | 0.11    |
| 46          | F      | SCC                  | 3              | 1 | 0 | 0.8  | 0.3 | 1.0     | 0.50    |
| 47          | M      | ADC                  | 2              | 1 | 0 | 0.2  | 0.8 | 0.1     | 0.23    |
| 48          | M      | SCC                  | 1              | 0 | 0 | 0.2  | 0.9 | 0.5     | 0.31    |
| 49          | F      | ADC                  | 4              | 2 | 0 | 0.7  | 0.2 | 0.0     | 0.06    |
| 50          | M      | ADC                  | 1              | 0 | 1 | 0.7  | 0.1 | 0.2     | 0.39    |
| 51          | M      | SCC                  | 1              | 2 | 0 | 0.8  | 0.3 | 0.6     | 0.28    |
| 52          | M      | SCC                  |                |   |   | 0.2  | 0.8 | 0.1     | 0.20    |
| 53          | M      | ADC                  | 1              | 0 | 0 | 0.5  | 0.6 | 0.4     | 0.22    |
| 54          | M      | ADC                  | 3              | 1 | 0 | 0.7  | 0.3 | 0.8     | 1.26    |
| 55          | M      | SCC                  | 2              | 0 | 1 | 0.8  | 0.1 | 1.0     | 5.15    |
| 56          | M      | ADC                  | x              | 3 | 1 | 0.6  | 0.1 | 0.7     | 0.18    |
| 57          | M      | ADC                  | 1              | 0 | 1 | 0.7  | 0.2 | 0.0     | 0.57    |
| 59          | M      | ADC                  | 3              | 0 | 0 | 0.3  | 0.6 | 0.8     | 0.25    |
| 60          | M      | LCC                  | 3              | 3 | 0 | 0.8  | 0.2 | 0.3     | 0.16    |
| 61          | M      | ADC                  | 3              | 2 | 0 | 0.8  | 0.2 | 0.7     | 0.68    |
| 62          | M      | LCC                  |                |   |   | 0.3  | 0.8 | 0.3     | 0.16    |
| 63          | M      | SCC                  | 3              | 2 | 2 | 0.9  | 0.4 | 1.0     | 0.55    |
| 65          | M      | SCC                  |                |   |   | 0.3  | 0.8 | 0.3     | 0.27    |
| 66          | M      | SCC                  |                |   |   | 0.2  | 0.9 | 0.2     | 0.24    |

Individual data of 62 patients on gender, histological subtype, TNM classification and expression levels of analysed markers. Expression level of protein markers P-gp, MRP and LRP/MVP is showed as percentage of positive cells (0.0 min – 1.0 max). Relative expression of miR-23b is showed as  $2^{-\Delta\Delta Ct}$  value. Value  $2^{-\Delta\Delta Ct} > 1$  means upregulation and  $2^{-\Delta\Delta Ct} < 1$  means downregulation of miR-23b. M – man, F – female.

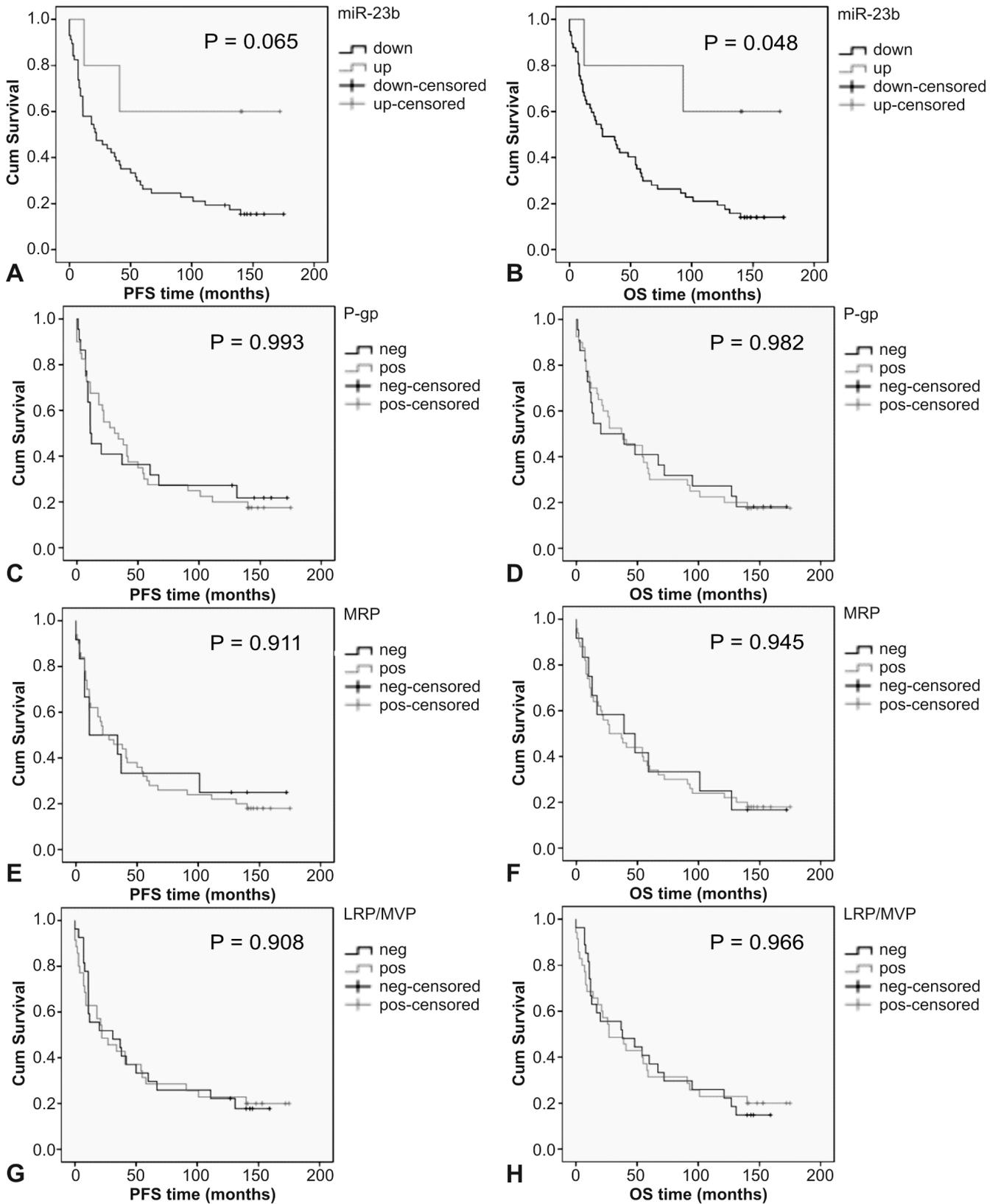


Figure 4. Survival analysis of miR-23b, P-gp, MRP and LRP/MVP in NSCLC patients. Kaplan-Meier curves represent PFS and OS of NSCLC patients and depend on the expression of miR-23b (A, B), P-gp (C, D), MRP (E, F) and LRP/MVP (G, H). PFS – progression-free survival, OS – overall survival.

meet the required criteria (PFS:  $P = 0.020$ ,  $\chi^2 = 5.400$ ; OS:  $P = 0.013$ ,  $\chi^2 = 6.185$ ). As a result, there was increased risk of death or relapse in patients with miR-23b downregulation and LRP/MVP expression from 0.1 to 1; and there was decreased risk of death or relapse in patients who had upregulated miR-23b (Figure 5). Association between risks and miR-23b with P-gp and MRP expression was not significant for modelling. Importantly, univariate Cox regression analysis of miR-23b indicated that there was a trend of association between miR-23b and risk of death or relapse in NSCLC patients (PFS:  $P = 0.087$ , HR = 3.451, CI (95%) = 0.836-14.246, Wald = 2.932; OS:  $P = 0.067$ , HR = 3.765, CI (95%) = 0.912-15.552, Wald = 3.357). Therefore, the combined model of miR-23b and LRP/MVP defines well the risk of death or relapse in NSCLC patients.

We also found that patients expressing P-gp from 0.1 to 1 with miR-23b downregulation had higher risk of cancer-related death compared to patients with miR-23b upregulation (RR = 2.595, CI = 0.521-12.920,  $\chi^2 = 5.430$ ,  $P = 0.02$ ; Figure 6). However, as mentioned above, combined expression of miR-23b with P-gp did not meet the criteria for modelling and no Cox regression model for these variables was prepared.

Our results showed that miR-23b, especially in combination with LRP/MVP, may serve as a prognostic biomarker for NSCLC patients. A larger cohort of patient samples is needed to be analyzed to confirm the results.

**Discussion**

The initial goal of this preliminary study was to determine the significance of miR-23b in NSCLC. Previous studies describe its dual role as an oncogenic or tumor-suppressor miRNA depending on cancer type [25]. The relative quantification of miR-23b in this study showed that miR-23b is mostly downregulated in NSCLC patient samples (57 out of 62 samples). Its downregulation has also been described in prostate, bladder and thyroid cancer. Higher levels of miR-23b are a good prognostic factor according to some authors [26, 28, 32]. The survival analysis data are in agreement with these studies showing that upregulation of miR-23b in tumors with its predominant downregulation is connected with better patient outcome ( $P_{PFS} = 0.065$ ;  $P_{OS} = 0.048$ ). There are also studies that present miR-23b as an oncogenic miRNA, where its upregulation in different tumors is associated with worse patient outcome [29-31]. The split role of miR-23b in different cancer types can be caused by targeting different molecules.

In tumors with miR-23b predominant downregulation it is known as a tumor-suppressor and targets mainly proteins responsible for proliferation and metastasis formation, such as AKT, Src kinase, ZEB1 or SNAIL [26-28]. In tumors with miR-23b upregulation, it functions as an oncogene and targets mainly proteins involved in apoptosis and metastasis suppression, such as Nischarin, VHL or PTEN [29-31].

Park *et al.* identified participation of miR-23b in chemoresistance [35]. Therefore, we decided to detect immunohistochemical expression of well-known proteins associated with MDR, namely P-gp, MRP and LRP/MVP. Expression of P-gp was detected in 40 (64.5 %), MRP in 50 (80.6 %) and LRP/MVP in 35 (56.4 %) patients. Our survival analysis showed no relationship between expression of these proteins and PFS and OS (P-gp:  $P_{PFS} = 0.993$ ,  $P_{OS} = 0.982$ ; MRP:  $P_{PFS} = 0.911$ ,  $P_{OS} = 0.945$ ; LRP/MVP:  $P_{PFS} = 0.908$ ,  $P_{OS} = 0.966$ ). Similar observations were also published by other authors [36-39]. On the other hand, there are also studies supporting the negative prognostic role of these MDR proteins in cancer patients [40-44]. Thus, we decided to establish Cox proportional hazard model for testing the possible prognostic significance of combined expression of these proteins and miR-23b and for assessing risk of death and relapse in our patients.

First, we found that patients expressing P-gp with miR-23b downregulation had higher risk of cancer-related death compared to patients with miR-23b upregulation (RR = 2.595,  $P = 0.02$ ). More importantly, our Cox proportional hazard model showed that the risk of death or relapse in patients with miR-23b downregulation increases together with expression of LRP/MVP and decreases in patients with miR-23b upregulation (PFS:  $P = 0.022$ , HR = 4.342; OS:  $P = 0.015$ , HR = 4.408). On the other hand, univariate analysis of miR-23b alone did not show any statistically significant association with patient's risk (PFS:  $P = 0.087$ , HR = 3.451; OS:  $P = 0.067$ , HR = 3.765). We are probably the first group interested in the prognostic role of miR-23b in combination with other known biomarkers in NSCLC. But available reports on the possible role of miR-23b in different cancer types support our observations. These authors found that miR-23b upregulation is associated with better patient's prognosis in tumors where it occurs as a tumor-suppressor [26, 28]. These preliminary results need to be validated in a larger cohort of cases.

**Table 5. Summary of expression of P-gp, MRP and LRP/MVP in patients with regard to miR-23b up- and downregulation.**

|              |      | P-gp  |       | MRP   |       | LRP/MVP |       |
|--------------|------|-------|-------|-------|-------|---------|-------|
|              |      | negat | posit | negat | posit | negat   | posit |
| miR-23b      | down | 20    | 37    | 10    | 47    | 26      | 31    |
|              | up   | 2     | 3     | 2     | 3     | 1       | 4     |
| <b>Total</b> |      | 22    | 40    | 12    | 50    | 27      | 35    |

**Table 6. Correlation analysis of expression of miR-23b and P-gp, MRP and LRP/MVP.**

| Protein | Chi-Square test |       | Mann-Whitney test |       |        |
|---------|-----------------|-------|-------------------|-------|--------|
|         | $\chi^2$ value  | P     | P                 | Z     |        |
| miR-23b | P-gp            | 0.048 | 0.826             | 0.599 | -0.526 |
|         | MRP             | 1.485 | 0.223             | 0.320 | 0.995  |
|         | LRP/MVP         | 1.227 | 0.268             | 0.115 | -1.576 |

The relationship between miR-23b and P-gp, MRP and LRP/MVP analysed by Chi-Square and Mann-Whitney tests.

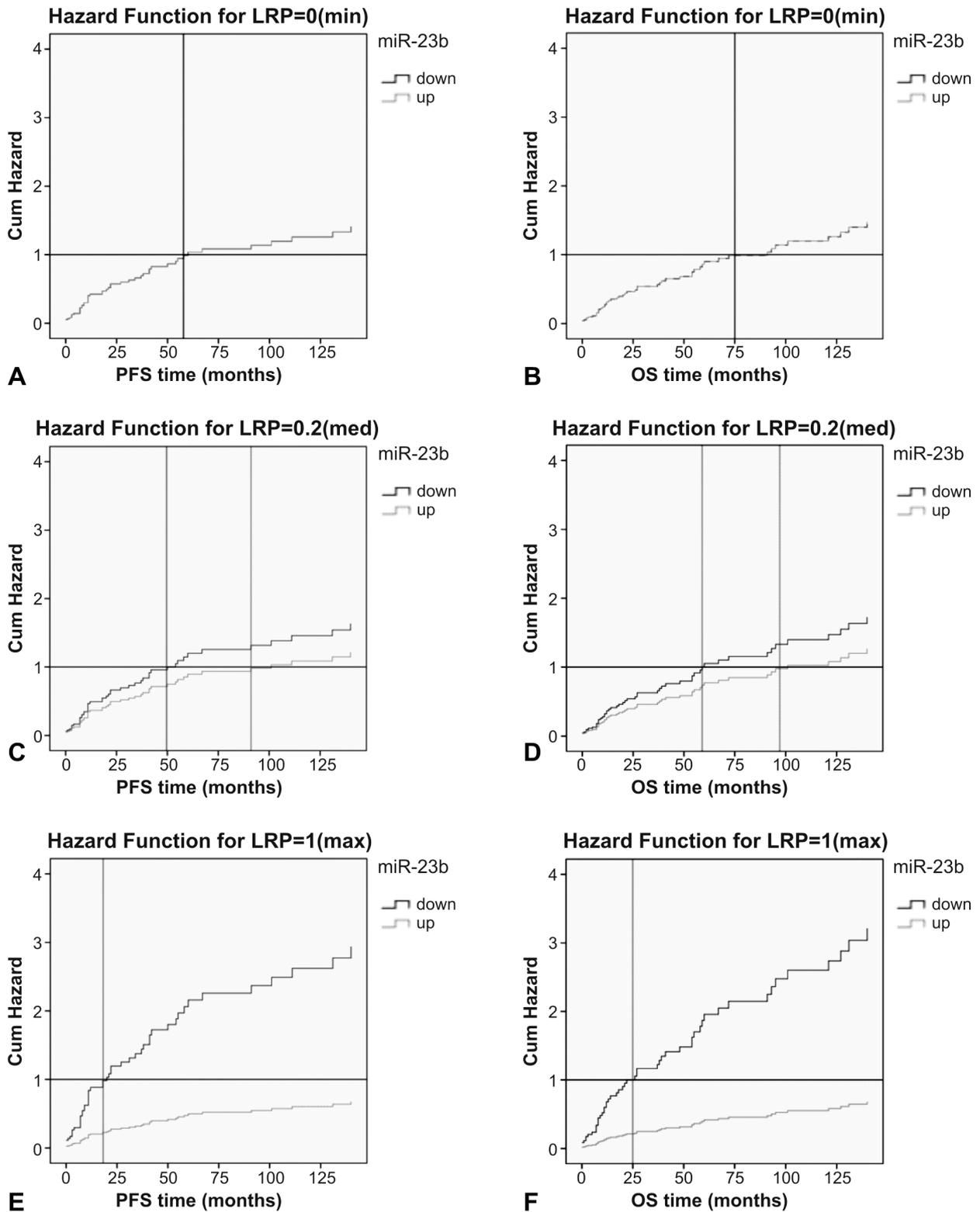


Figure 5. Cox regression hazard model of miR-23b and LRP/MVP expression in NSCLC patients. Hazards of death and relapse between miR-23b down- and upregulation groups are the same when LRP/MVP expression is 0 (A, B). Hazard is higher in the group with miR-23b downregulation in comparison to the group with miR-23b upregulation when LRP/MVP expression is 0.2 (median) (C, D). The difference between the hazards of miR-23b down- and upregulation groups is the highest when LRP/MVP expression is 1 (max) (E, F).

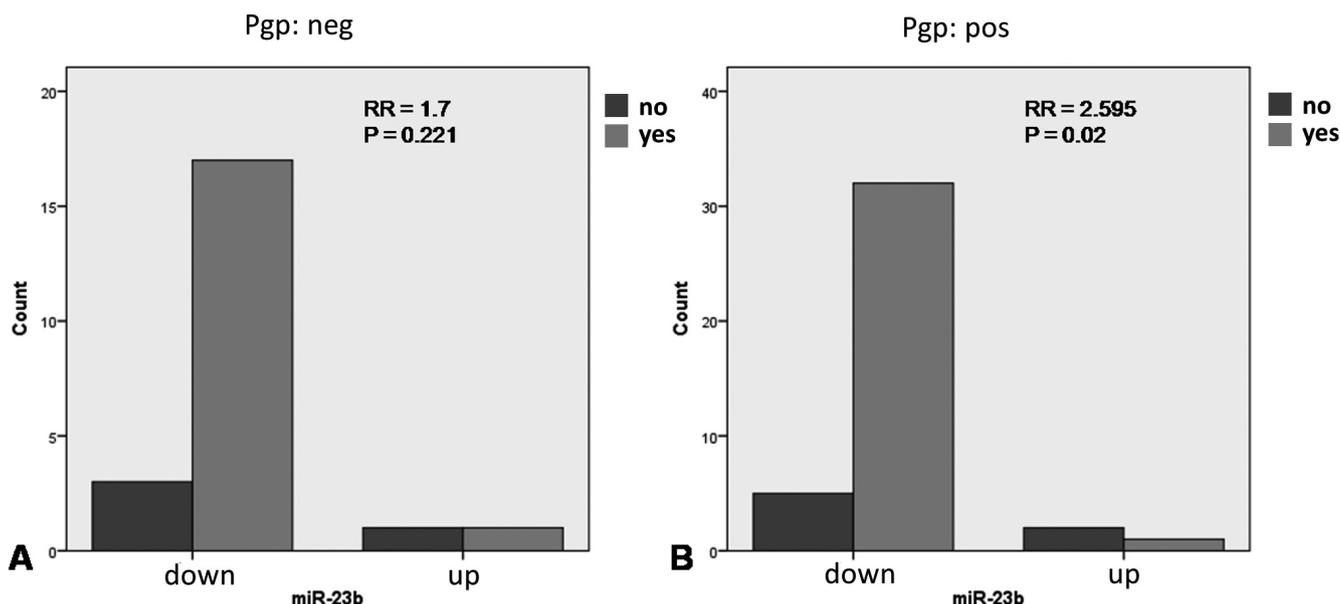


Figure 6. Odds ratio of P-gp and miR-23b expression in NSCLC patients. Patients with miR-23b downregulation had higher risk of cancer-related death compared to patients with miR-23b upregulation in the case of P-gp expression from 0.1 to 1.

**Conclusion**

In this study, we determined the possible role of miR-23b which is very often deregulated in different cancers and its role in NSCLC has not been fully established yet. The results suggest that miR-23b may function as a tumor-suppressor in NSCLC as it was often downregulated in advanced tumors and patients had better outcome when it was upregulated. Our results also showed that detection of miR-23b levels together with P-gp and LRP/MVP expression may be suitable for assessing the prognosis and risk in NSCLC patients. According to the regression model, the risk of death or relapse in NSCLC patients with downregulated miR-23b increases together with LRP/MVP expression and decreases in patients with upregulated miR-23b.

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