

Correlation Between Promoter Hypomethylation and Increased Expression of Syncytin-1 in Non-Small Cell Lung Cancer

This article was published in the following Dove Press journal:
International Journal of General Medicine

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Introduction: Syncytin-1 is a human endogenous retroviral (*HERVW*) envelope protein, which has been implicated in trophoblast and cancer cell fusions as well as in immunomodulatory functions. We investigated syncytin-1 expression and promoter methylation in non-small cell lung cancer (NSCLC) and the adjacent, para-carcinoma tissues. In addition, the correlation to patient survival differentiation of between 5-year survival and death group was analyzed.

Methods: Survival ratio was calculated by Kaplan-Meier survival curve. Death risk assessment was executed by Cox risk regression model. The 5'-LTR methylation level of *HERVW* promoter was detected by EpiTYPER method.

Results: Syncytin-1 expression in NSCLC tissue was found to be significantly higher than in para-carcinoma tissues. Moreover, the 5-year survival group has a lower syncytin-1 expression than the death group. Clinical stage and the percentage of syncytin-1 positive cells were top risk factors according to Cox ratio risk regression model analysis. While the methylation level of the 5'-LTR in *HERVW* gene promoter was relatively lower in NSCLC than para-carcinoma tissues, the methylation status of a CpG-2 site overlapping the Oct-1 binding site was found to be an important element potentially involved in the epigenetic regulation of *HERVW* gene expression.

Conclusion: These findings suggest that syncytin-1 could be a biomarker for the diagnosis/prognosis of NSCLC, and further studies are required to elucidate the exact role of syncytin-1 in the development of NSCLC as well as the underlying molecular mechanism for syncytin-1 function and regulation.

Keywords: non-small cell lung cancer, syncytin-1, epigenetic regulation, prognosis, DNA methylation

Introduction

More than 25% tumor mortality is caused by lung cancer worldwide,¹ and 85% of lung cancers belong to non-small cell lung cancer.² According to an updated report from the American Cancer Society (ACS), it was estimated that in 2020, 1,806,590 new cancer cases and 606,520 cancer deaths were projected to occur in the United States alone.³ Approximately 75% of lung cancers are diagnosed in late stages that are associated with a low 5-year survival rate of 15.6%.^{4,5} In these late stage cases, treatments including surgery, radiotherapy or chemotherapy are of very limited efficacy, highlighting an urgent need for early diagnosis and treatment.

Syncytin-1 is a functional glycoprotein encoded by the *env* gene located at chromosome 7q21-22. As a member of the human endogenous reverse transcription

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virus W gene family,^{6,7} syncytin-1 is highly expressed in human placenta and several carcinoma tissues. Maliniemi et al. reported syncytin-1 expression in 15 of 30 epididymic malignant lymphoma.⁸ Sun et al. reported significantly increased expressions of syncytin-1 in archaeocytes, lymphocytes, granulocytes and monocytes during acute myelocytic leukemia.⁹ Larsson et al. reported that 38% of 165 premenopausal node-negative breast cancers are positive for syncytin-1 expression, and this positivity correlates with the poor outcomes of patients.¹⁰ Strissel et al. disclosed that syncytin-1 is over-expressed in late-stage and poorly differentiated endometrial carcinoma rather than the early-stage and highly differentiated one.¹¹ The expression of syncytin-1 in non-small cell lung cancer tissues remained poorly understood.

It is well recognized that genetic changes such as site mutation, deletion, translocation, and/or amplification as well as epigenetic changes including alterations in histone modifications, patterns of DNA methylation and/or microRNA can all contribute to the carcinogenesis of lung cancers.^{1,12} Multiple genetic mutations including translocation of *ALK* gene, mutation of *EGFR* gene and rearrangement of *ROS-1* gene have been identified and the oncogenic mechanisms well characterized.¹³ Epigenetic changes, through their impact on gene transcription, may lead to activation of oncogene and silence of tumor suppressor genes.¹⁴ *HERVW* gene is not only overexpressed in various types of tumors, recent studies indicated that the gene products may be involved in modulation of cell cycle and cell apoptosis in placental trophoblasts.¹⁵ Its expression and epigenetic regulation in lung cancers, however, remains uninvestigated.

In this study, syncytin-1 expression in carcinoma and para-carcinoma tissues of NSCLC is detected by immunohistochemistry. The 5'-LTR methylation of *HERVW* promoter is determined by EpiTYPER method. Correlation of syncytin-1 expression and carcinogenesis of NSCLC is analyzed. These studies may provide novel information regarding its involvement in the carcinogenic mechanism of NSCLC. Such knowledge could be useful for the improvement of diagnosis, prognosis and management of NSCLC cases.

Materials and Methods

Patients and Tissue Samples

Thirty patients clinically diagnosed as NSCLC from Jan 1999 to Oct 2001 in Qilu Hospital, Shandong

University, were included in this study, of which 23 were male and 7 female, ages ranging from 37–75 (57.87±1.72 in average), 14 glandular and 16 squamous, 12 TNM stage I, 6 stage II and 12 stage III, 15 with lymphatic metastasis and 15 without. None of the patients received radiotherapy or chemotherapy before surgery. The inclusive criterion was: Survived for more than 1 month after surgery and did not die of any cause other than lung cancer during the follow-up time of 1–78 month (34 on average). Tissue samples were collected from pulmonary primary lesions with pathological characteristics identified under microscope. This study was authorized and administrated by the Ethics Committee of Qilu Hospital, Shandong University. Para-carcinoma refers to the adjacent regions 1–2 cm from the edge of carcinoma.

Immunohistochemistry

Carcinoma and para-carcinoma tissues removed in surgery were fixed with 10% neutral formalin for 12–24 h and embedded in paraffin. Tissues were sectioned into slides in thickness of 6 µm, dewaxed in xylene for 5 min twice, and dehydrated with 100%, 95%, 85%, 75% alcohol for 60 each. Tissue sections were boiled in EDTA repairing fluid for 2.5 min and rinsed with PBS three times for antigen retrieval after cooling. Endogenous peroxidase was blocked with 100 µL 3% hydrogen peroxide by incubation at 37°C for 10 min before being rinsed with PBS three times. Antibody solution is obtained by 1:100 dilution from the rabbit anti-human syncytin-1 monoclonal antibody (GeneTex, USA). The tissue slides were exposed to 50 µL of antibody solution at room temperature for 2 h. After extensive rinsing with PBS, the tissue slides were stained with the secondary antibody (Zhongshan Jinqiao, Beijing) by incubation at 37°C for 30 min. Color development was performed with DAB solution (Zhongshan Jinqiao, Beijing) for 3–5 min. After counter staining with hematoxylin, the slides were dehydrated, cleared, and mounted for microscopic observation.

Evaluation of Staining

Syncytin-1 protein in cytoplasm and cell membrane was positively stained in brownish color and the nuclei was in blue color. The percentage of positive cells was calculated, and semi-quantitative analysis was performed by categorization of the staining density into four classes: negative (0 positivity), weakly positive

(lower than 25% positivity), moderately positive (25–50% positivity) and strongly positive (more than 50% positivity).

EpiTYPER Methylation Detection

The paraffin-fixed tissues were sliced into 10 μm thickness, and 5–10 tissue slides were used for each DNA extraction. PCR primer sequences were designed by the EpiDesigner software: 5'-TTTTTTTTGGGATGAGGGTAAA and 3'-TTAAACAAACCCAAACTTAACC. PCR amplification was executed at 94°C for 4 min as initial denaturalization, followed by 45 cycles of denaturalization at 94°C for 20 s, annealing at 56°C for 30 s, extension at 72°C for 1 min, with hydrosulfite DNA as templates. The PCR products were visualized by 1.5% agarose gel electrophoresis under 160 V for 20 min as follows (Figure 1). PCR products were digested with SAP at 37°C for 20 min, 85°C for 5 min, and dotted into MassArray sampler for mass spectroscopy detection. The results were analyzed with the EpiTYPER software.

Statistical Analysis

The syncytin-1 staining positivity of NSCLC carcinoma and para-carcinoma were compared with Student's t-test using the SPSS 17.0 statistical software, and $P < 0.05$ was considered statistically significant. Survival curve was constructed with the Kaplan-Meier method and intro-curve comparison was performed with the Log rank test. Prognosis was evaluated by Cox ratio risk recurrence model. The 5'-LTR methylation of *HERVW* promoter was analyzed by mass spectroscopy and EpiTYPER software. The average methylation ratio and standard deviation was calculated with the SPSS 17.0 statistical software, and compared with non-parametric rank sum test and Mann-Whitney *U*-test. $P < 0.05$ was considered statistically significant.

Results

Comparison of Syncytin-1 Staining Positivity Between NSCLC Carcinoma and Para-Carcinoma Tissues

Syncytin-1 protein staining positivity was 96.7% (29/30), with 65.5% (19/29) strongly positive (Figure 2A) and 34.5% (10/29) moderately positive (Figure 2B) in carcinoma tissues, and 33.3% lowly expressed in para-carcinoma tissues (Figure 2C). Syncytin-1 positive cell rate in carcinoma tissues (35.41% \pm 0.22%) was significantly higher than in para-carcinoma tissues (2.17% \pm 0.03%) ($P < 0.01$) (Figure 3). High expression is defined as higher than 25% positive cell rate.

Comparison of Syncytin-1 Expression Between 5-Year Survival and 5-Year Death Group

Syncytin-1 expression positivity was 44.67% \pm 0.21% in the 5-year death group and 23.30% \pm 0.16% in the 5-year survival group. The former was significantly higher than the latter ($P < 0.01$) (Figure 4).

Survival Analysis

Five-year mortality of syncytin-1 high-expression group was 73.7% and the low-expression group was 27.3%, with a significant difference between the two groups ($P < 0.05$). The average survival period of the low-expression group was 64 months while the high-expression group was only 28 months, and the difference was statistically significant ($P < 0.01$) (Figure 5) according to the Kaplan-Meier survival curve analysis. Clinical stage and positive cell percentage were found to be lethal factors of NSCLC based on the Cox ratio risk recurrence model (Table 1).

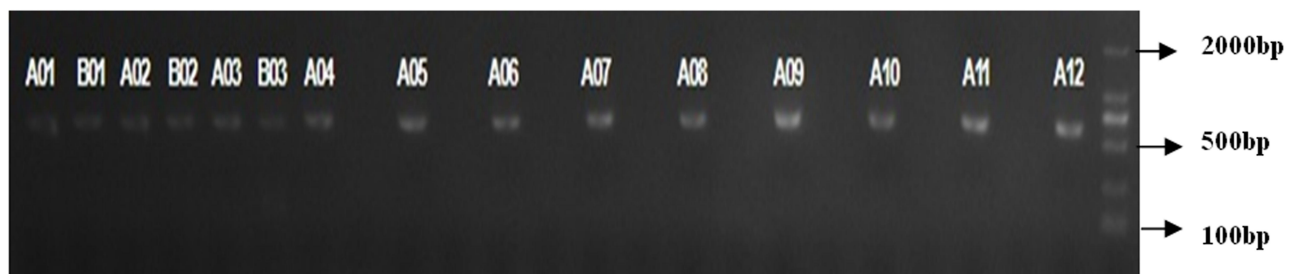


Figure 1 Detection for the PCR products of tissue DNA by 1.5% agarose gel electrophoresis. The marker: DL2000 bands are 2000 bp, 1000 bp, 750 bp, 500 bp, 250 bp, and 100 bp from top to bottom.

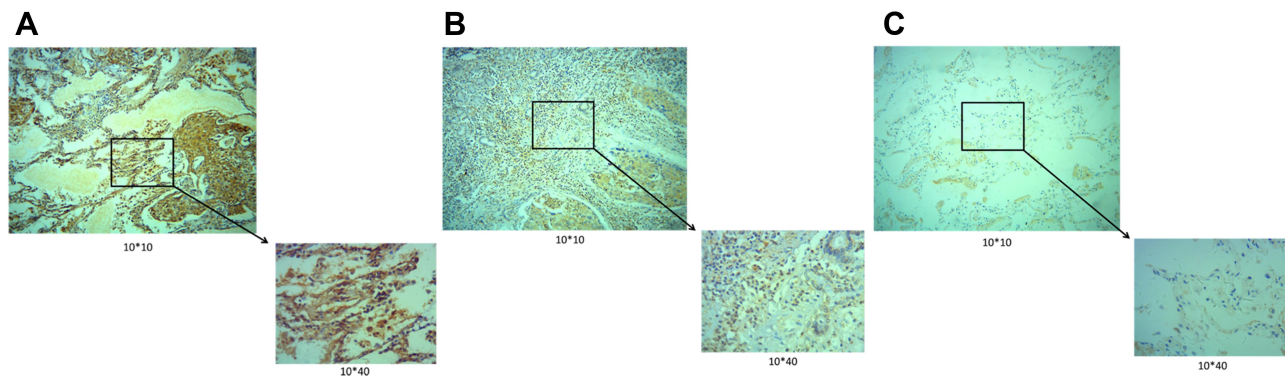


Figure 2 Syncytin-I staining in cancer tissue and para-cancerous tissue. Tumor cells were evident with strong syncytin-I staining (A), moderate syncytin-I staining (B) in cancer tissues and nearly no staining of syncytin-I in para-carcinoma tissues (C).

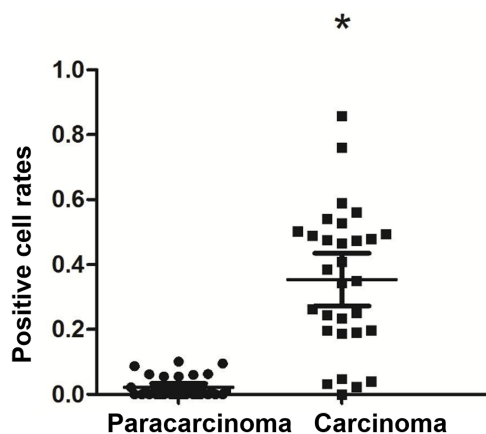


Figure 3 Syncytin-I positive cell rate in NSCLC tissues and para-carcinoma tissues. The syncytin-I positive cell rate in NSCLC tissues was higher than that in adjacent tissues. *Represents $P < 0.05$.

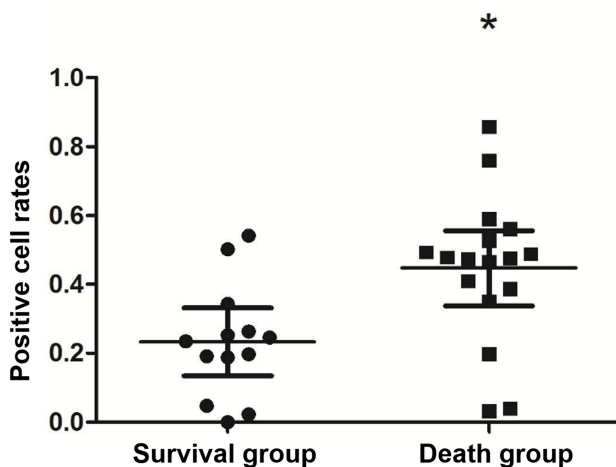


Figure 4 Syncytin-I positive cell rate in 5-year survival group and 5-year death group. The syncytin-I positive cell rate of the carcinoma tissues in the 5-year death group was higher than that in the 5-year survival group. *Represents $P < 0.05$.

Comparison of Total Methylation Ratio and CpG2 Site Methylation Ratio

The total methylation ratios of NSCLC tissues and para-carcinoma tissues were $77.50\% \pm 10.19\%$ and $78.87\% \pm 11.54\%$, respectively (Figure 6), with no significant difference ($P > 0.05$). The CpG2 site methylation ratios of NSCLC tissues and para-carcinoma tissues were $81.91\% \pm 12.38\%$ and $94.33\% \pm 4.93\%$, respectively, showing a significant difference between the two groups ($P < 0.05$) (Figure 7, Table 2) according to the non-parametric rank sum test and Mann–Whitney *U*-test of two independent samples.

Discussion

Lung cancer was particularly prevalent during the twentieth century due to the popularity of tobacco consumption, especially in developed countries where lung cancer ranks at the top of the morbidity and mortality list. The carcinogenesis of lung cancer is a complicated process influenced by environmental, genetic, epigenetic, physical, chemical, immunological, and infectious factors, e.g., the activation of oncogenes and inactivation of suppressor genes. An earlier diagnosis and therapy would lead to a more optimistic prognosis. The 5-year survival ratio of local lung cancer or bronchogenic carcinoma is 54.8%, while this ratio drops sharply to 27.4% and even 4.2%, respectively, for regional and widespread lung cancers.¹³ Hence, a novel lung cancer biomarker could be essential for developing tools for early diagnosis. Our results suggest that the increased syncytin-I expression and/or the alteration of DNA methylation in

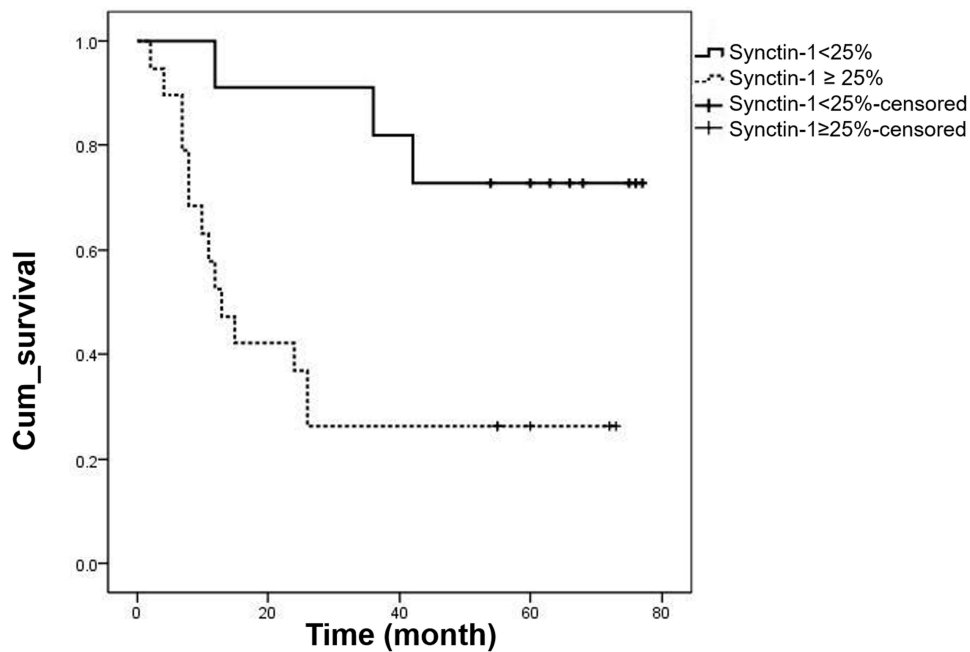


Figure 5 Survival curve of high expression and low expression of Syncytin-1. Kaplan-Meier survival curve analysis showed that the syncytin-1 positive cell rate low expression group had a higher recurrence-free survival rate.

the 5'-LTR of *HERVW* promoter could be potential biomarkers for the diagnosis of lung cancers.

Larsen et al.¹⁶ performed a retrospective analysis on 140 colon and rectum cancer cases. It was found that syncytin-1 was commonly expressed in both carcinoma of colon and rectum, and the rectum carcinomas showed high syncytin-1 expression levels as well as low 5-year survival ratios. On the other hand, Larsson et al.¹⁰ reported that syncytin-1 expression could be an independent positive indicator for breast cancer prognosis since patients with higher syncytin-1 expression in their tumors had a higher non-palindromic survival ratio. In this study, higher expression of syncytin-1 in NSCLC tissue than in

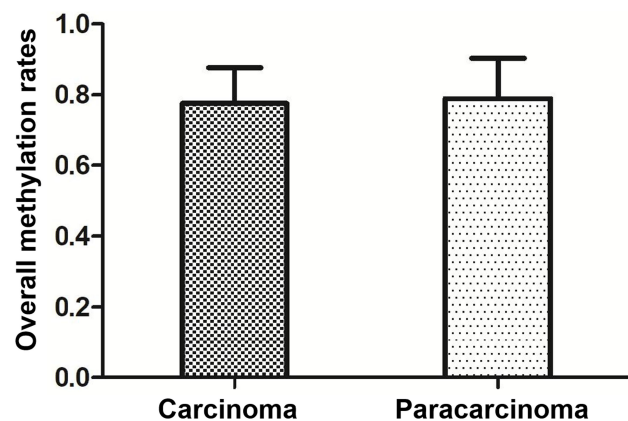


Figure 6 Comparison of overall methylation rates in NSCLC tissues and paracarcinoma tissues. The overall methylation rate of 5'-LTR in the promoter region of *HERVW* in NSCLC tissues was lower than that of adjacent tissues.

Table I Multivariate Cox Regression Analysis Results of Non-Small Cell Lung Cancer Patients

| Characteristics | HR | 95% CI | P-value |
|----------------------------------|-------|--------------|---------|
| Age, years (<60/≥60) | 0.639 | 0.179–2.273 | 0.489 |
| Sex (male/female) | 0.678 | 0.176–2.617 | 0.573 |
| Syncytin-1 (<25%/≥25%) | 7.824 | 1.138–53.814 | 0.037* |
| Lymph node metastasis (N0/N1/N2) | 0.449 | 0.160–1.263 | 0.129 |
| Stage (I/II/III) | 8.158 | 2.211–30.093 | 0.002* |
| Depth of tumor (T1/T2/T3) | 1.258 | 0.506–3.126 | 0.621 |

Note: *P<0.05.

para-carcinoma tissue as shown by immunohistochemistry is consistent with previous studies in other carcinoma tissues. However, the syncytin-1 expression level was higher in the 5-year death group than the survival group, and the K-M survival curve also demonstrated that the survival ratio in the low-expression group was 27.3% (3/11) with an average survival time of 64 months, while the ratio in the high-expression group was 73.7% (14/19) with an average survival time of only 27 months. This finding suggested that for NSCLC, a high syncytin-1 expression level is associated with a worse prognosis. Among the

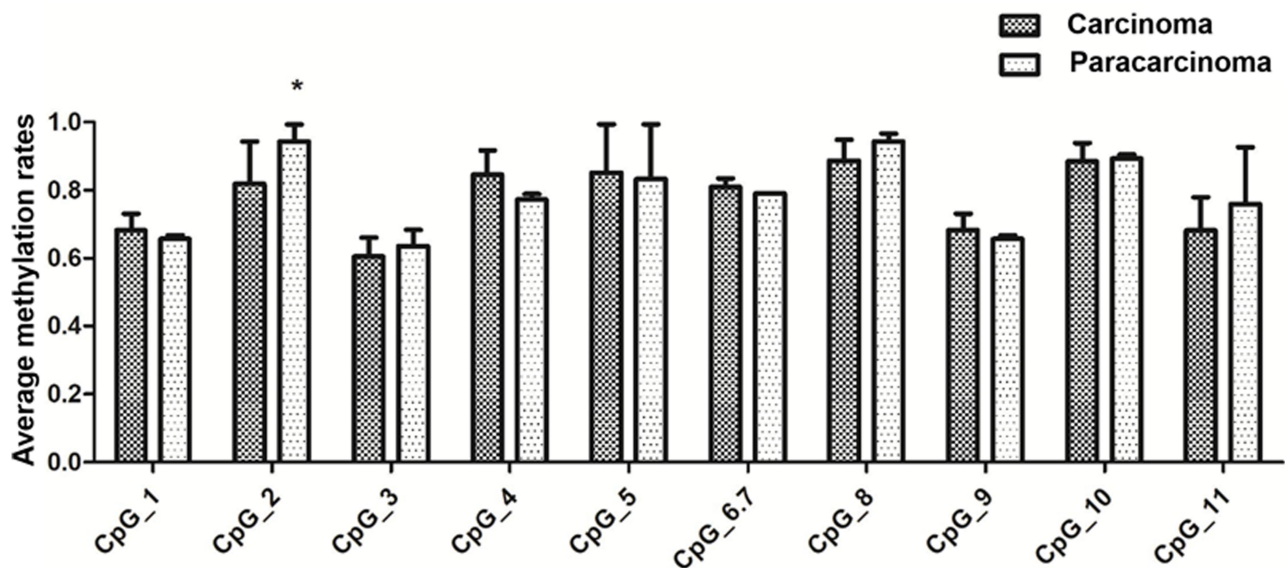


Figure 7 Comparison of average methylation rates of CpG sites in NSCLC tissues and para-carcinoma tissues. The average methylation rate of 10 CpG loci of 5'-LTR in the promoter region of *HERVW* in carcinoma tissues and para-carcinoma tissues was detected, and the difference in methylation rate of CpG2 loci was statistically significant. *Represents $P < 0.05$.

factors considered, including age, gender, syncytin-1 positive cell percentage, clinical stage and TNM stage, and clinical stage, syncytin-1 positive cell percentage was found to be the most important factor of NSCLC mortality according to the Cox ratio risk recurrent model. Hence, syncytin-1 could be a novel biomarker for NSCLC diagnosis and prognosis.

Table 2 Comparison of Methylation Rates of CpG Sites in Non-Small Cell Lung Cancer Tissues and Adjacent Tissues

| Gene Locus | NSCLC Tissues M (%) | Adjacent Tissues M (%) | Z-Vvalue | P-value |
|------------|---------------------|------------------------|----------|---------|
| CpG_1 | 68.25 | 65.67 | -0.729 | 0.536 |
| CpG_2 | 81.91 | 94.33 | -2.171 | 0.031* |
| CpG_3 | 60.50 | 63.67 | -0.651 | 0.536 |
| CpG_4 | 84.58 | 77.33 | -1.592 | 0.136 |
| CpG_5 | 85.16 | 83.33 | -0.292 | 0.840 |
| CpG_6.7 | 80.91 | 79.00 | -1.574 | 0.136 |
| CpG_8 | 88.75 | 94.33 | -1.447 | 0.180 |
| CpG_9 | 68.25 | 65.67 | -0.729 | 0.536 |
| CpG_10 | 88.50 | 89.33 | -0.507 | 0.633 |
| CpG_11 | 68.16 | 76.00 | -0.795 | 0.448 |

Note: * $P < 0.05$.

Abbreviation: M, methylation rate.

Syncytin-1 mediates the fusion of chorionic trophoblasts (CT) to form syncytiotrophoblasts (ST), and the reduction in syncytin-1 expression plays an essential role in the pathogenesis of intrauterine growth restriction (IUGR), preeclampsia (PE) and HELLP syndrome.¹⁷ Besides the function in cell fusion, decreased syncytin-1 expression led to cell apoptosis as observed in preeclampsia.^{18,19} Syncytin-1 was also reported to promote cell proliferation through activation of TGF- β 1 and TGF- β 3, to mediate immunosuppression during adaptation to pregnancy.^{20,21} Over 75% of bladder urothelial carcinoma has up-regulated syncytin-1 expression, which contributes to the proliferation of urothelial cells.²² Moreover, in the neuroblastoma cell line, manipulation of syncytin-1 led to abnormal regulation of the cAMP signaling pathway, and toxic excitation of neurons and subsequent neural system diseases.²³ Thus, non-fusogenic activities of syncytin-1 may be involved in the regulation of a variety of functions in tumor cells. More studies are required to verify if NSCLC could be one of such tumors.

The high expression of syncytin-1 appears to be associated with the low-level methylation of 5'-LTR promoter. Matouskova et al. argue that syncytin-1 expression in trophocytes is triggered by demethylation of the promoter region resulting in proliferation and differentiation of syncytiotrophoblast cells.²⁴ Zhuang et al. reported syncytin-1 down-regulation and increased 5'-LTR methylation in the preeclamptic placenta.²⁵ Zhou et al. reported that syncytin-1 over-expression correlated with a decreased promoter

methylation level in endometrial cancers.²⁶ Benesova et al. compared spermocytoma and nonseminoma and found a low-level methylation of *HERVW* promoter in the former and high-level methylation in the latter.²⁷ In endometrial cancers, hypomethylated 5'-LTR was associated with up-regulated expression of syncytin-1.¹¹

At least two components, that is, U3 region of 5'-LTR promoter and its upstream TSE function to regulate the transcription of *HERVW* gene owing to the CAAT box and octamer protein binding site of U3 region and 5'-LTR upstream GCMA binding site, which interacts with GCMA transcription factor to increase syncytin-1 expression level in BeWo and JEG3 choriocarcinoma cell lines.^{21,28,29} In this study, 10 CpG sites of 5'-LTR promoter of *HERVW* gene in NSCLC and para-carcinoma tissues have been investigated (Figure 8). The result demonstrates that *HERVW* promoter in NSCLC tissues has a relatively lower level of methylation than in para-carcinoma tissues, especially in the CpG2 site, suggesting a site-specific methylation of *HERVW* promoter in NSCLC tissue. Our findings are generally consistent with the above observations, all point to an epigenetic regulation of syncytin-1 expression in tumor cells.

Further analysis of the CpG2 site located 95 nt downstream from the transcriptional start site found it overlaps with an Oct-1 transcription factor binding site. Oct-1 is a DNA-binding transcriptional factor regulating a large number of target genes including housekeeping genes and specific genes involved in immune, endocrine, and

neural functions.³⁰ High levels of Oct-1 expression have been found in somatic stem cells and cancer stem cells, and gastric, prostatic, cervical, and esophageal cancers.³¹ *HERVW* promoter demethylation may result in an increased binding by transcriptional factors such as Oct-1, to induce the expression of syncytin-1 protein. Thus, the CpG2 site could be a key epigenetic component mediating epigenetic and/or non-epigenetic regulation mechanisms of syncytin-1 expression under different sorts of pathological conditions.

The study had several limitations. Firstly, only one methylation site was found to show significant differences, which led to insufficient evidence on the relationship between methylation and protein expression, which may be related to the small number of samples or stale samples. Therefore, in the subsequent study, we will collect fresh tissue samples to carry out a multi-center joint study.

In this study, an over-expression of syncytin-1 was found in NSCLC tissues compared with adjacent tissues. The overexpression of syncytin-1 was associated with a worse prognosis of patient survival. A low-methylation level in the 5'-LTR promoter region of the *HERVW* gene, especially a specific CpG site overlapping with an oct-1 binding site was identified as a key element for the regulation of syncytin-1 expression. Although the precise molecular mechanism remains elusive, the observations suggest that syncytin-1 could be a novel biomarker for lung cancer diagnosis/prognosis, and further studies along this line

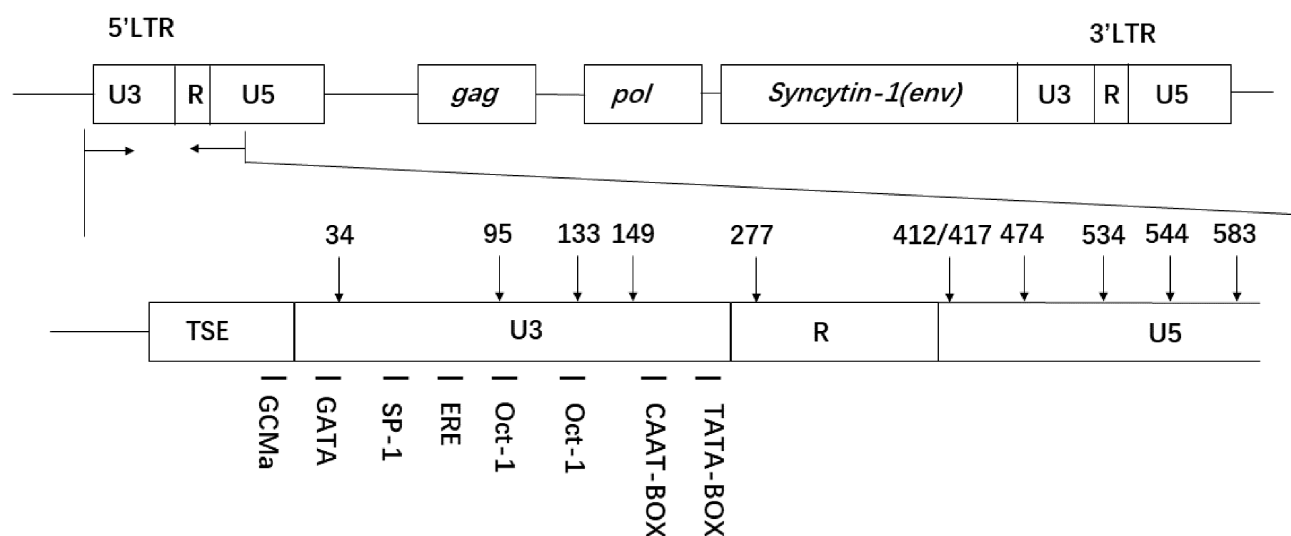


Figure 8 Schematic map of the *HERVW* gene. The downward arrow and number indicate the 10 CpG sites and position in the methylated fragment, respectively. The TSE is the trophoblast-specific enhancer region located directly upstream of the 5'-LTR U3 region. In addition, important cis-acting elements are highlighted below the methylated fragment.

may be useful for improving the management of lung cancers.

Abbreviations

NSCLC, non-small cell lung cancer; MF, muscarinic fungoides; CT, chorionic trophoblast; ST, syntrophoblast; IUGR, intrauterine growth restriction; PE, preeclampsia.

Ethical Approval

Ethical approval for this project was obtained from the Ethics Committee of Qilu Hospital, Shandong University. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent

Written informed consent was obtained from all individual participants included in the study.

Acknowledgments

This study was supported by grants from Shandong Provincial Nature Science Foundation (No. ZR2015CM030), Shandong Key Research and Development Program (No. 2016GSF201169).

Disclosure

The authors declare that they have no conflicts of interest.

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