microRNA-146 up-regulation predicts the prognosis of non-small cell lung cancer by miRNA in situ hybridization

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Non-small cell lung cancer (NSCLC) accounts for approximately 70% of all lung cancer-related deaths worldwide. Prognostic markers are essential for the early detection of lung cancer in patients. In this study, we first identified microRNA146 (miR-146) expression in cancer cell lines using miRNA in situ hybridization (MISH) and confirmed the accuracy of MISH using q-RT-PCR. In addition, two different systems, BCIP/NBT and ELF, were used to detect the signal for a comparative analysis of the specificity of MISH. Compared to the BCIP/NBT system, the ELF detection system was more effective for MISH. Furthermore, we detected the expression of miR-146 in NSCLC tissues (43 cases) and normal tissues (32 cases). Based on our results, we can conclude that miR-146 is more highly expressed in cancer tissue than normal tissue (t-test, P < 0.05) and that miR-146 can predict the prognosis of NSCLC by MISH. Our findings preliminarily demonstrate that MISH can be applied as a molecular diagnostic tool to determine the expression and localization of miRNAs in cancer tissues and that miR-146, determined by MISH, predicts the prognosis of NSCLC patients.

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Introduction

Non-small cell lung cancers (NSCLCs) are the leading cause of cancer mortality, and the overall 5-year survival of NSCLC patients is not more than 15% (Jemal et al., 2009). If one can diagnose NSCLC at an early stage, the survival rate of patients can be effectively improved. Therefore, some specific early molecular markers are urgently needed for the detection of lung tumors. microRNAs (miRNAs) may open the door to the early molecular diagnosis of NSCLC and allow risk stratification based on miRNA profiles (Cho, 2011; Mendell, 2005; Xing et al.).

miRNAs are small non-coding RNAs of approximately 20–23 nucleotides that can regulate cancer cell processes by annealing to the 3′ UTRs of target genes (Griffiths-Jones et al., 2006). Some miRNAs are important as oncogenes or anti-oncogenes (Cho et al., 2011; He et al., 2005); many of these can facilitate the development of lung cancer as has been reported in previous studies (Bishop et al.). Meanwhile, lung cancer-related miRNA profiles have been established (Gee et al.; Hayashita et al., 2005; Vaporidi et al., 2012). For example, the increased expression of miR-146 was correlated with poor prognosis in lung cancer patients.

Currently, analyses of miRNA expression are usually performed by q-RT-PCR. Northern blotting using miRNA probes and microchip array technology (Hu et al.; van Rooij, 2011). However, these techniques cannot detect the localization of miRNAs in a tissue or single cell; miRNA in situ hybridization (MISH) can accomplish this (Neely et al., 2006). Perhaps more importantly, there are great differences between the expression profiles of miRNAs in cancer cells and non-cancer cells. Our previous study demonstrated that MISH can be applied to detect the expression of miRNA375 in paraffin-embedded esophageal cancer tissue (J. Li et al.; Y. Li et al.). In this work, we overcame the technical difficulty that the melting temperature (Tm) of the miRNA probe hybridization complex is too low to allow for its detection. Additionally, we further analyzed miR-146 to confirm its value. Several studies have reported that miR-146 is associated with lung cancer (Perry et al., 2009), but further details on the specific subcellular compartmentalization of miR-146 have not been reported. It is not possible to use RT-PCR or other methods to detect the expression of a miRNA in single cells or at low copy expression (Lu and Tsourkas, 2009).

Therefore, our study focuses on MISH and verifies the accuracy of this technique using q-RT-PCR. Briefly, we describe the MISH method to determine miR-146 expression in cultured cells and paraffin-embedded tissues and use it to detect the expression of miR-146 in NSCLC. The primary objective of this work is to enrich the molecular detection of miRNAs in cancer tissues.
Materials and methods

Cell culture and tissue treatment

Non-small-cell lung cancer cell lines ACC212102 and SCC211441 (established by our lab) and KYSE140, KYSE180, KYSE510, and HKESC1 cell lines were cultured in DMEM with 10% FBS supplemented with l-glutamine. The cells were cultured on glass cover slides at 37 °C in a 5% CO2 environment. When the cells reached 70%–80% confluence, they were immediately fixed in 10% formalin for 2 h and MISH was performed on glass cover slides as described below.

Clinical specimen collection

The expression of miR-146 was evaluated in a total of 43 non-small cell lung cancer and 32 matched normal adjacent lung tissue samples by miRNA in situ hybridization (MISH). The samples included 20 lung adenocarcinoma samples, 17 squamous cell carcinoma samples, 6 adeno-squamous carcinomas, and no large-cell carcinomas. All the specimens were collected from patients in the Department of Thoracic Surgery, Sun Yat-Sen University Cancer Center, Guangzhou, China from October 2009 to July 2012 with patient consent and institutional review board approval.

ISH probes

An oligonucleotide probe, complementary to the hsa-miR-146b-5p (miR-146) probe, was purchased from Exonbio Lab (Guangzhou, China). The sequence of the probe is 5′-AGCCTATGGAATTCAGTTCTCA-3′; the 5′ and 3′ ends were modified with digoxigenin (DIG). Some of these bases were modified with 2-fluorine. A probe with the sequence 5′-AGCGTATGGAATTCAGATCTCA-3′ served as control probe.

![Fig. 1. MISH results are consistent with RT-PCR. (1) miRNA expression was analyzed in a series of cell lines by MISH, according to the data transformation as shown in A and B, as described above. The gray mean is shown in C. (2) In addition, the expression of miR-146 in these cell lines was quantified using RT-PCR, shown in a histogram (D). The expression of miR-146 by MISH detection is positively correlated with the data obtained from RT-PCR. The correlation coefficient (R² = 0.93) of the two methods is significant (P < 0.05, E and F).](image-url)
In situ hybridization (ISH) for miR-146 was performed on fixed paraffin-embedded sections as previously described (J. Li et al.; Y. Li et al.). The MISH procedure was carried out as follows: The paraffin wax was removed from the tissue sections with fresh xylene three times, and then the samples were washed in PBS and RNase-free water for 3 min. Next, the tissues were digested with protease in 0.1 N HCl for 10 min and 0.1% PBS for 5 min. We recommend proteinase K to treat tissue that has been fixed for more than six months. The proteinase K concentration is 20 mg/ml, and working dilutions are 1:100 in PBS. Notably, proteinase K may lead to over-digestion, which can cause loss of tissue morphology. After digestion, the samples were immersed in RNase-free water and air-dried, and then they were pre-hybridized and hybridized with miRNA probe or control probe at a concentration of 0.5 μM. The samples were incubated at 37 °C for 24 h; then two stringent washes were carried out in 2 × SSC containing Tween-20 at 25 °C for 2 min and 2 × SSC for 2 min at room temperature. ISH was performed twice on each slide.

Detection systems

The ELF detection (Paragas et al., 1997) kit (Invitrogen, San Diego, CA) utilizes substrate cleavage by phosphatase to produce a yellow-green fluorescent signal at the site of enzymatic activity. The ELF precipitate is up to 40 times brighter than common probes directly labeled with fluorophores. The probe was labeled by DIG combined with anti-Dig conjugated biotin, and detection was carried out according to the manufacturer’s specifications. The slides were counterstained with Hoechst 33342 (1 μg/ml).

The NCIP/NBT detection system was purchased from Roche Diagnostics. The probe was labeled with DIG combined with anti-Dig conjugated alkaline phosphatase (AP), and the cells were stained with NCIP/NBT. The nuclei of cells were stained by Nuclear Fast Red.

Quantification of MISH images, analysis and statistics

To investigate the accuracy of MISH in cell lines, a qRT-PCR method was adopted to quantify the expression of miRNA. To validate if MISH accurately reflects the expression of miR-146 in cell lines, we wanted to analyze whether the expression level of miR-146 detected by MISH correlated with the RT-PCR results. To accomplish this, we compared the mean gray value of miR-146 MISH per cell with the values obtained by quantitative RT-PCR. The MISH images were analyzed using image J software using the Tool/image/split of the color menu option. The results are shown in Fig. 1; the green signals were used to analyze the gray value, and the gray signals were then measured using the mean

Fig. 2. Comparison between the specificity of the ELF and BCIP/NBT detection systems. (1) The upper panels show the BCIP/NBT detection system results; A and B show lung cancer tissue and pericancerous tissues, respectively. (2) D shows high expression of miR-146 obtained by the ELF detection system; the photographs in E and B display pericancerous tissues, in which the arrow indicates a few cells specifically expressing miR-146. C and F are controls. All images are magnified 400×. These results demonstrate that the ELF-MISH system can better detect the miR-146 than the BCIP/NBT detection system.
gray value program in the software. The cells were imaged with an Olympus BX51 fluorescence microscope. The nucleus was stained by Hoechst to evaluate the co-localization of miRNA.

Results

Analysis of the accuracy of miR-146 ISH using qRT-PCR

To investigate whether MISH can be applied to accurately determine the expression of miRNAs and whether MISH results accurately reflect the real expression of miRNAs (Fig. 1A), we compared the results of MISH experiments to those of RT-PCR. First, we measured the MISH spots, which corresponded to an individual miR-146, and next, we counted 20–40 cells in cell culture and obtained the mean gray value of miR-146 expression using image J software (the format of Fig. 1A is identical to that of B). A quantification of miR-146 using RT-PCR in the same cell lines (Fig. 2C) revealed that MISH could reflect the relative expression of miRNA in single cells (Fig. 1D). However, in cases with high miR-146 expression, we could not determine the actual expression level because it was difficult to discern the fluorescent gray value using the software. Nonetheless, the approximate miRNA copy numbers could still be estimated in the single cells by MISH because there is a linear correlation between the expression of miRNA by RT-PCR and the mean gray value (the total cellular fluorescence in a single cell). The R² value measuring this correlation is significant (Figs. 1E and F, P < 0.05, R² = 0.9329).

Confirming the specificity and sensitivity of miRNA ISH using EF and BCIP/NBT detection systems

To further validate the specificity and sensitivity of the miR-146 probe for MISH, we evaluated two detection systems: the EF and BCIP/NBT detection systems, which were previously used for mRNA ISH (Millet et al., 1996) or IHC. Both adapted detection systems can reveal the miR-146 expression signals and detect single cells expressing miR-146, as shown in Fig. 2. The representative images in Figs. 2A and B were obtained with the BCIP/NBT detection system, and the images in Figs. 2D and E were obtained with the ELF detection system for miRNA ISH. From the data, we can deduce that the BCIP/NBT detection system is less effective than the ELF detection system. Alternatively, the results suggested that MISH with the ELF kit is superior to the conventional BCIP/NBT detection system in cancer tissue and that the sensitivity of the ELF detection system is dramatically higher than that of BCIP/NBT. Unfortunately, the shortcoming of ELF is also noticeable; the ELF kit has a high background (Fig. 2D), which could lead to difficulties in differentiating the miRNA expression. Figs. 2C and F represent control samples.

The up-regulation of miR-146 in NSCLC is associated with poor prognosis

To further apply and optimize the method for diagnosis, we examined the expression of miR-146 in 43 lung NSCLC and 32 matched adjacent normal lung tissue samples using MISH. The expression of miR-146 is higher in NSCLC than in adjacent normal lung tissue (Fig. 3A). It has

Fig. 3. High expression of miR-146 is associated with poor prognosis. (1) A representative photo of miR-146 expression in cancer tissue and the peritumoral tissue. The “Ca” indicates a cancer nest, which is separated from the peritumoral tissue by the dotted line (A); miR-146 is more highly expressed in the cancerous tissue than in the peritumoral tissue. (2) The expression of miR-146 in cancer tissue by MISH. Among 43 cases, 31 cases show high miR-146 expression. The results demonstrate that the expression of miR-146 is up-regulated in NSCLC carcinoma tissues (A and B). (3) The up-regulation of miR-146 expression was significantly associated with poor overall survival (C, P < 0.05, Log risk = 10.56) in NSCLC patients.
been reported that the expression of miR-146 (analyzed by RT-PCR) is associated with the clinical prognosis of NSCLC, and this is consistent with our results. The score standard was defined as “high” and “low” according to the representative Figs. 3A and B. The correlation between miR-146 down-regulation and clinical–pathological features was analyzed by SPSS 16.0 software. The up-regulation of miR-146 is significantly correlated with the overall survival rate (one-way ANOVA, P < 0.05) but is not associated with other clinical characteristics (table not shown). To further investigate the prognostic value of miR-146 in NSCLC, Kaplan–Meier analysis was carried out; the high expression of miR-146 can be associated with overall survival in the NSCLC cohort (Fig. 3C, P = 0.003, Log rank = 10.56). The median expression of miR-146 was used to classify the patients into two halves. The group with high miR-146b expression had a significantly worse overall survival rate (16.8 months) compared to the group with low miR-146b expression (57.7 months).

**Discussion**

The expression of miRNAs varies with the development of lung cancer (Izzotti et al., 2009), and most of these miRNA variations emerge during tumorigenesis (Landi et al.; Lu et al., 2005). The methods of determining miRNAs included q-RT-PCR, Northern blotting and miRNA chip arrays. FISH is a traditional method to detect DNA localization or miRNA expression. However, there are few reports using FISH for diagnosis in cancer tissue (Hermansen et al.; Sempere and Korc). However, the advantages of subcellular localization are currently being re-evaluated in light of new molecular factors such as miRNAs. In our previous study, we optimized a MISH method by testing miR-375 in ESCC (J. Li et al.; Y. Li et al.). Herein, we further verified the expression of miR-146 in lung cancer tissue. Consistent with our expectations, miR-146 is highly expressed in NSCLC and can specifically detect the high expression of miR-146 in lung cancer but not in normal tissue.

The data from MISH have shown that miR-146 is highly expressed in NSCLC tissue, which is consistent with previous studies using RT-PCR. We compared the results of two different MISH detection systems including ELF and BCIP/NBT; the results were similar, but the ELF detection method is better suited for MISH because it can identify single expressing cancer cells. In addition, miR-146 was also tested using RT-PCR in cell lines in this study, and the results are consistent with MISH. According to this study, high expression of miR-146 is associated with poor outcomes in NSCLC. Therefore, a MISH method to detect miR-146 may be a valid tool for predicting the patient survival rate in lung cancer.

MiRNAs are more stable than mRNAs, which enables them to be readily detected in formalin-fixed paraffin-embedded tissue (Kong et al.). Moreover, our study shows that miRNA ISH is an effective way to determine the expression of a miRNA. In summary, we have demonstrated that miR-146 expression can be accurately detected by MISH and these experimental data can be used to analyze patient prognosis. MISH may provide a unique method for miRNA detection and may predict the prognosis of lung cancer (Raponi et al., 2009). In addition, we provided support for the use of MISH in studying miRNA or in molecular diagnosis. Additional studies should continue work on these aspects of MISH technology.

**Conflicts of interest statement**

The authors disclose no potential conflicts of interest.

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