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miRNAs, a potential target in the treatment of Non-Small-Cell Lung Carcinomas ☆

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ABSTRACT

Lung cancer is a serious public health problem and Non Small Cell Lung Carcinoma, NSCLC, is particularly resistant to current treatments. So it is important to find new strategies that are active against NSCLC. miRNA is implicated in cancer and may be implicated in NSCLC. Our team has been working on two genes HEF1, a gene implicated in different functions of cell cycle and B2, a large non-coding RNA (nc RNA). These two genes have the same localisation: chromosome 6 and locus p24–25. nc RNA B2 may be involved in the regulation of HEF1. Firstly, we examine a bank of different human miRNAs known to interact with exons of HEF1. HEF1 and B2 were overexpressed *in vitro* by treating NSCLC-N6 with the cytostatic molecule A190, and carried out qRT-PCR for the expression of miRNA. Secondly, using specific software, we sought for structures originating from the B2 RNA sequence which might interact with HEF1 and assessed their expression.

This strategy enabled us to confirm firstly that known miRNAs that can interact with exons of HEF1 are expressed in NSCLC-N6 cells. More precisely this strategy highlighted overexpression of one miRNA, hsa-miR-146b, listed in miRbase. The second step of the studies highlighted the expression of miRNA, potentially sequences originating from B2 in the NSCLC-N6.

This miRNA overexpressed might be one of the regulators of the gene HEF1 and consequently implies on the carcinogenesis of lung cancer. So in the future it could be a potential and an innovative way to find a new strategy for the treatment of lung cancer.

1. Introduction

Lung cancer is a serious public health problem with 278,000 new cases being reported each year in France. Non Small Cell Lung Carcinomas (NSCLC) are particularly resistant to current treatments. Thus, given the low survival rate of patients with this type of cancer, it is important to find new molecules that are active against NSCLC. Chemotherapy for NSCLC, which offers the best response rate (although only about 30%), currently uses molecules such as Permetrex, which acts on the signalling of apoptosis in association with molecules which act on tubulin and/or microtubules (Zinner et al., 2004). It is thus essential to direct research towards new cellular therapies which will target other mechanisms. Many potential molecular targets are currently being studied: for example, those which inhibit the *ras* gene in order to block cell proliferation (Wong, 2009) or block tyrosine kinase pathways by acting on the Epidermal Growth Factor receptors (EGF receptors) (Kotsakis and Georgoulias, 2010), or block angiogenesis by acting on the vascular endothelial growth factor (vEGF) (Niu and Chen, 2010). Another approach that seems promising is to inhibit the translation of genes involved in proliferation by the induction of microRNA (miRNA). In fact, the miRNAs are small endogenous non-coding RNAs which can regulate gene expression and may promote carcinogenesis. For example, miR-17–92 is a miRNA implicated in lung cancer, which may target Phosphatase and TENsin homolog (PTEN) and/or Rabbit antihuman RB2, but this has not been demonstrated (Hayashita et al., 2005). The miRNAs may also be used as prognostic factors. In fact, the overexpression of the miRNA LET-7 in the lung is an indicator of poor prognosis (Calin and Croce, 2006; Esquela-Kerscher and Slack, 2006; Takamizawa et al., 2004; Zhang et al., 2007).

The team of Cimmino has shown that the expression of miR-15a and miR-16-1 regulates the B-cell lymphoma 2 (*BCL-2*) gene negatively, which enables cell apoptosis to be induced in chronic lymphocytic leukaemia. The miRNAs thus seem to be a very promising molecular target, in the treatment of cancer, especially NSCLC (Cimmino et al., 2005).

Abbreviations: NSCLC, Non Small Cell Lung Cancer; HEF1, Human Enhancer of Filamentation 1; EGF receptors, Epidermal Growth Factor receptors; vEGF, vascular Endothelial Growth Factor; PTEN, Phosphatase and TENsin Homolog; BCL-2, B-Cell Lymphoma 2; PCR, Polymerisation Chain Reaction; QRT PCR, Quantification Reverse transcription Polymerisation Chain Reaction; miRNA, Micro RiboNucleic Acid.

☆ This study is an original research, which has not been previously published and has not been submitted for publication elsewhere while under consideration.

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For several years, we have been researching anticancer treatments that are more specific for cancer cells and less toxic for normal cells. With this objective, our team is studying two new molecular targets. These two genes are overexpressed when NSCLC-N6 cells are treated with the molecule A190, a molecule which has a cytostatic profile which induces an antitumour activity (Moreau et al., 2008). *HEF1* is involved in different cellular functions such as proliferation, differentiation and apoptosis, while the gene *B2* is a large non-coding RNA which overlaps part of *HEF1* (Malleter et al., 2010). These new molecular targets, *HEF1* and *B2*, may provide new means of regulation in NSCLC.

Given that the miRNAs are implicated in cancer as regulators of oncogenes such as miR-15a (Cimmino et al., 2005), it is possible that they are also involved in NSCLC. In fact, the non-coding RNA *B2*, in view of its size of 54 kb, may be able to intervene in the regulation of *HEF1* by splitting B2 into miRNA. With this in mind, we induced overexpression of *HEF1* and *B2 in vitro* by treating NSCLC-N6 cells with A190, then tested the expression of miRNA.

Secondly, using specific software, we sought for structures originating from the *B2* RNA sequence which may interact with the mRNA of *HEF1*.

2. Materials and methods

2.1. Cell lines and cultures

Two cell lines were used in this study, A549 and NSCLC-N6, originating from an adenocarcinoma and an epidermoid lung cancer, respectively. The NSCLC-N6 is a cell line derived from a NSCLC of a previously untreated patient (moderately differentiated classified as T2N0M0) (Roussakis et al., 1991). The A549 line was obtained from the ATCC (reference CCL-185) (Giard et al., 1973) and is known to have a wild type *p53* gene, while NSCLC-N6 has a mutant *p53* gene, similar to tumours *in situ*.

These cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 enriched with 100 IU of penicillin and 100 μg/ml of streptomycin, 2 mM of glutamine and 5% foetal bovine serum. Cell culture plates were maintained in humidified incubators at 37 °C in a 5% CO_2 atmosphere. NSCLC-N6 has a cell doubling time of 48 h *in vitro*, and that of A549 cells is 24 h.

2.2. Cell synchronisation in the M phase

During mitosis, cell shape and adhesion change dramatically. Indeed, this phase of mitosis induces a depolymerisation of the microtubules (Sawin and Endow, 1993; Suzuki and Takahashi, 2003) and a loss of the cytoskeleton and adhesion to the support. Thus, the cells just lie at the bottom of the culture flask throughout mitosis. It is therefore easy to isolate them by slightly stirring the culture medium, taking off the cells and suspending them. The cells are then salvaged by slow centrifugation and placed in a new culture flask.

2.3. Treatment of NSCLC-N6 cells

A total of 5×10^4 cells/ml were incubated in the presence of 39.4 μM A190 for 60 h; wells without A190 acted as a control.

2.4. Extraction of total RNA

Total RNA was extracted from NSCLC-N6 and A549 cells using TRIzol® reagent (Invitrogen, CA, USA) and chloroform according to the supplier's recommendations. Cell extracts were centrifuged at 12,000*g* for 15 min at 4 °C. The aqueous phase containing RNA was transferred to a fresh tube. An equal volume of 70% ethanol was added to the aqueous phase and mixed by vortexing. Samples were then transferred to an RNA spin cartridge supplied with the TRIzol® plus RNA Purification Kit (PureLink™ Micro-to-Midi™ Total RNA Purification System, Invitrogen, Applied Biosystems) in order to remove DNA contamination. Finally, RNAs were eluted with RNase-free water.

The quality and concentration of purified RNAs were assessed using UV absorbance at 260/280 nm and samples were run on 1% agarose gel in order to assess their quality. RNAs were stored at −80 °C.

2.5. DNase treatment

To remove genomic DNA prior to reverse transcription PCR, TURBO ™ DNase was used. If the RNA concentration was >200 μg/ml, samples were diluted to 10 μg nucleic acid/50 μl. 10× TURBO DNase buffer was added to sample to 1× final concentration before adding 2 U of TURBO DNase to 10 μg RNA in a 50 μl reaction mixture. Samples were incubated at 37 °C for 30 min. DNase Inactivation Reagent was then added, the mixture was incubated for 2 min and then centrifuged at 10,000*g* for 1.5 min. The supernatant, which contained RNA, was transferred to fresh tubes.

2.5.1. Quantification of microRNA

First we select hsa-miR with the software in open access: http://microrna.sanger.ac.uk/sequences/. One of the criteria to select the miRNA is to present a homology with HEF1 gene. These sequences were tested by quantitative PCR on the cDNAs obtained from the total RNA (2 μg) from cells synchronised treated or not and isolated by a TRIzol protocol. Then we have done a RT PCR specific of the miRNA. This Reverse transcription consisted a step of polyadenylation and reversely transcribed for use in two-step quantitative RT-PCR using the NCode miRNA First-Strand Synthesis and qRT-PCR kits (Invitrogen) according to the manufacturer's instructions. The resulting cDNA was subjected to real-time qRT-PCR using the NCode universal reverse primer in conjunction with a sequence-specific forward primer for hsa-miR-146b (TGAGAACTGAATTCCATAGGCT 22 bp). A master mix was prepared for each PCR reaction, which included SYBR GreenER™ qPCR SuperMix of Invitrogen, forward primer, Universal qPCR Primer, ROX Reference Dye and 1 μl template cDNA. β-actin was used for the standard curve. The reactions were placed in a 96-well plate using a preheated real-time PCR instrument (ABI 7900HT). The programme was 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 58 °C for 60 s. After cycling, the reaction mixture was maintained at 4 °C until further analysis.

The expression of these miRNAs was standardised by measuring its ratio to the reference gene of β-actin. These qPCR were carried out 3 times to confirm the results.

In a second time, we have selected 12 potential sequence of miRNA from B2 sequence which is chosen by the software: http://www.dharmacon.com/DesignCenter/DesignCenterPage.aspx. The criteria to select potential sequence of miRNA are to present a homology with HEF1 and to be induced in B2. The strategy and step to analyse this sequence were the same as before.

2.6. Statistical analysis

The *t* test is done to analyse the results of qRT-PCR.

3. Results

3.1. Expression of human miRNAs in NSCLC-N6 cells

In order to determine the presence of miRNA in the cellular model, we promoted the induction of miRNA in the cells. Our aim was to show, by qRT-PCR, the expression of miRNA in NSCLC-N6 cells, synchronised and treated or not with A190 for 60 h. The miRNAs known to come from the human genome, potentially expressed in our NSCLC-N6 cellular model, were chosen strategically.