Gene 506 (2012) 355–359

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

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

Marine Malleter, Catherine Jacquot, Bénédicte Rousseau, Christophe Tomasoni, Marcel Juge, Alain Pineau, Vehary Sakanian, Christos Roussakis *

Nantes University, Nantes Atlantique University, IICIMED/ERT-A0902, Cancer du Poumon et Cibles Moléculaires (CPCM), UFR Sciences Pharmaceutiques, Nantes, France

ARTICLE INFO

Article history:
Accepted 6 June 2012
Available online 23 June 2012

Keywords:
miRNA
Large non coding RNA
HEF1
B2
A190
Hsa-miR-146

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.

© 2012 Elsevier B.V. All rights reserved.

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.

* Corresponding author at: Université de Nantes, Nantes Atlantique Université, IICIMED/ERT-A0902, Cancer du Poumon et Cibles Moléculaires (CPCM), UFR des Sciences Pharmaceutiques, 1 rue Gaston Veil, Nantes, F.44 035 cedex 1, France. Tel.: +33 2 5112 5672; fax: +33 2 5112 5690.

E-mail address: christos.roussakis@univ-nantes.fr (C. Roussakis).

0378-1119/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.gene.2012.06.008

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.

This was done by using the databases available on the web, such as miRbase (http://microrna.sanger.ac.uk/sequences/), which list previously identified miRNAs. We selected only those miRNAs known and isolated from humans (prefixed 'hsa' according to the nomenclature). However, the origin was not the only characteristic used. In fact, we also chose miRNA on the basis that they could come from the *B2* RNA sequence and could hybridise with one of the exons of the *HEF1* gene. Thus ten miRNAs were chosen whose characteristics and sequences are presented in Table 1.

The expression of the different human miRNAs was then tested by qRT-PCR in NSCLC-N6 cells, synchronised and treated or not with the molecule A190 for 60 h. After several quantitative PCR assays were carried out on the cDNA from the specific reverse transcription of miRNAs from the total RNA of treated and untreated NSCLC-N6 cells, the expressed miRNAs are shown in Fig. 1, statistical analysis in Table 3 and highlighted in Table 1. Among those tested, only five hsa miRNAs were expressed: hsa-miR-146b, hsa-miR-633, hsa-miR-423, hsa-miR-1305, and hsa-miR-1306. According to the unilateral *t* test, the expression of hsa-miR-146b, hsa-miR-633 and hsa-miR-423 was significantly greater in cells treated with A190 for 60 h than in untreated cells, while the expression of miRNAs hsa-miR-1305 and hsa-miR-1306 miRNAs showed no significant difference ($p<0.05$).

Moreover, the expression of hsa-miR-146 in treated cells was the most significant of all ($p<0.002$). Based on these statistics and its homology with exon 4 of the HEF1 gene, this miRNA was chosen for further study.

3.1.1. Potential sequences of miRNA that could intervene in the regulation of the HEF1 gene

Thus, *HEF1* might be regulated by miRNAs. In order to verify this hypothesis, we determined the potential sequences of miRNA that display all the characteristics for becoming a mature miRNA. These characteristics are managed by the software (http://www.dharmacon.com/DesignCenter/DesignCenterPage.aspx) freely accessible on internet. The 12 sequences chosen may intervene in the regulation of *HEF1* as they are homologous to the exons of *HEF1* according to our bioinformatic study. These sequences are presented in Table 2.

As the *B2* RNA sequence extends more than 10 kb upstream of exon 1 of the *HEF1* gene, it seemed important to select several possible miRNAs in this region (upstreams 1, 2 and 3). Once the expression of these was tested, we determined if they belonged to *B2* RNA.

These sequences were tested by quantitative PCR on the cDNAs obtained from the RNAs of synchronised NSCLC-N6 cells treated by A190. The RNAs underwent DNase treatment were reverse transcript with specific miRNA RT-PCR. The results and only those miRNAs presenting a significant expression are shown in the figure below (*cf.* Fig. 2 and statistical analysis Table 4).

We thus tested these 12 sequences by quantitative PCR under the conditions previously used for the miRNAs from the miRbase database. Among them, four were expressed: two were complementary to the sequence upstream of the *HEF1* gene, the third was complementary to exon 7 of *HEF1* and the last to exon 2 of *HEF1*.

Table 1
Different hsa miRNAs tested by real-time PCR.

Hsa	Sequence of HSA	*HEF1* exon	Expressed
hsa-miR 422A	5′-GCCTTCTGACCCTAAGTCCAGT-3′	3	No
hsa-miR 1324	5′-GAAAGTGCATAGAATTCTGTCTGG-3′	3	No
hsa-miR 146b	5′-AGCCTATGGAATTCAGTTCTCA-3′	4	Yes
hsa-miR-1305	5′-TCTCTCCCATTAGAGTTGAAAA-3′	4	Yes
hsa-miR-671	5′-CTCCAGCCCCTCCAGGGCTTCCT-3′	5	No
hsa-miR-633	5′-TTTATTGTGGTAGATACTATTAG-3′	5	Yes
hsa-miR-423	5′-AAAGTCTCGCTCTCTGCCCCTCA-3′	6	Yes
hsa-miR-578	5′-ACAATCCTAGAGCACAAGAAG-3′	6	No
hsa-miR-1306	5′-CACCACCAGAGCCAACGT-3′	7	Yes
hsa-miR-548h-4	5′-GACAAAAACCGCGATTACTTTT-3′	7	No

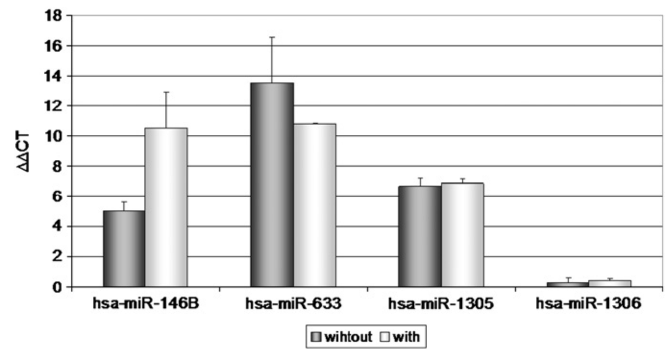

Fig. 1. The expression of different miRNAs selected from a data bank tested by qRT-PCR on NSCLC-N6 cells treated or not with 39.4 μM of A190 molecule for 60 h. The results are expressed as a ratio of mRNA quantity of the miRNA tested than of β-actin (control gene).

According to the statistical Mann–Whitney test, the expression of the three miRNAs complementary to the zone upstream of exon 1 of *HEF1*, as well as that of the miRNA 7B, was not significantly different between treated and untreated cells.

Moreover, miRNA 2A, complementary to exon 2 of *HEF1*, presented a very significantly greater expression in cells treated with A190 compared to untreated cells.

4. Discussion

From a therapeutic point of view, the use of miRNAs is at an experimental stage. Nevertheless, they represent a real hope in the treatment of various pathologies. In particular, the literature shows that the expression of miRNAs is deregulated in numerous types of cancer. Deregulation of some miRNAs is a factor of poor prognostic of cancers such as miR-155, miR-203, miR-210, and miR-222 overexpressed in pancreatic cancer (Greither et al., 2010). However, miRNAs may also be regulators of apoptosis: for example, downregulation of miR-15a and miR-16-1 targeting BCL-2 induces apoptosis in chronic lymphocytic leukaemia. In addition, miRNA LET-7 can be used as a prognostic factor or a regulator of tumorigenesis in lung cancer. It is located on chromosome 22 and its overexpression can inhibit the growth of lung cancer cells *in vitro*. In fact, it targets the *RAS* gene. Moreover, its reduced expression is associated with a lower postoperative survival rate (Takamizawa et al., 2004). Similarly, the miRNA hsa-miR-146b is overexpressed in thyroid cancer alongside miR-221 and -222 which are indicators for thyroid cancer and which also seem to inhibit cellular migration in glioma (Nikiforova et al., 2008; Xia et al., 2009). In fact, this miRNA acts by reducing the expression of a matrix metalloproteinase gene. Thus, the miRNAs clearly have regulatory functions which may be as diverse as their origins (Cimmino et al., 2005).

Table 2
Potential miRNAs designed and tested by quantitative PCR.

Name	Hypothetical sequence of miRNA	*HEF1* exon	Expressed
miR-1a	5′- TTTTCCTACACTAGTTAAG-3′	1	No
miR-1b	5′- TTATACTTCATTTCGCAGC-3′	1	No
miR-2a	5′- TATACCTCTTGTTCTTGGG-3′	2	Yes
miR-3a	5′-TATCTGGATGGGTACTCGT-3′	3	No
miR-4a	5′-TATCTCTCCCACTGGAACT-3′	4	No
miR-5a	5′-TCTCTCAATAGCTGTGTCT-3′	5	No
miR-6a	5′-TTTCCAATAGCTCTTTCTG-3′	6	No
miR-7a	5′-TTCTCCACGGGCTTTGTA-3′	7	No
miR-7b	5′-TTTCTGTACAGTTTATGTC-3′	7	Yes
miR-a1	5′-TTTCCCTCCACTAATATTT-3′	Upstream of exon 1^{HEF1}	Yes
miR-a2	5′-TAGTTCTGCATCTATGAGC-3′	Upstream of exon 1^{HEF1}	Yes
miR-a3	5′-TGTCTCTAGAAGTTTGCCG-3′	Upstream of exon 1^{HEF1}	No

Table 3
The table indicates the t-test results for each miRNA tested.

	Hsa-miR-146	Hsa-miR-633	Hsa-miR-1305	Hsa-miR-1306
t Value	4.273	2.535	1.200	1.502
Critical value of t (one-sized)	0.003	0.032	0.148	0.103

Table 4
The table indicates the t-test results for each miRNA.

	miRNA 2a	miRNA upstream 1	miRNA upstream 2	miRNA 7B
t Value	4.813754614	10.000	10.000	7.000
Critic value of t (one-sized)	0.00428093	0.014	0.014	0.333

Our team is working on the *B2* gene, a large (54 kb) non-coding RNA which we discovered in 2004 (Jacquot et al., 2004). It is located on chromosome 6 in the 6p24–25 region. It overlaps the *HEF1* gene, forming a single exon from exon 5 to 10 kb beyond exon 1 of *HEF1*. Its homology with *HEF1* makes it particularly interesting (Malleter et al., 2010) as this gene is involved in a large number of cellular processes such as proliferation, differentiation and apoptosis (Law et al., 1996, O'Neill and Golemis, 2001, Sakai et al., 1994). Thus we believe that *B2* RNA, due to these characteristics, may be able to regulate the *HEF1* gene miRNAs. In this study, we wished to investigate the overexpression of miRNA in the NSCLC-N6 experimental model and thus the involvement of miRNAs in NSCLC and cell proliferation.

We first recorded the expression of some of the known miRNAs in synchronised NSCLC-N6 cells treated with A190. The ten miRNAs already known and listed in the miRbase database that we tested all exhibited homology with the *HEF1* gene and originated from the human genome (type hsa). The expression of these miRNAs was analysed by qRT-PCR specific to miRNA. Secondly, we verified that the *B2* RNA sequence included structures favourable for the formation of miRNA. This analysis was carried out, on each zone of *B2* RNA which overlapped an exon of the *HEF1* gene. It soon became clear that a large number of potential sequences of miRNA were possible in each of these zones. Based on the statistical score of forming miRNA, given by the software for each sequence, we chose 12 potential sequences from *B2* to be tested by specific qRT-PCR.

The analysis of the ten known miRNAs has revealed the expression of five of them in synchronised cells treated or not with A190 for 60 h: hsa-miR-146b, hsa-miR-633, hsa-miR-423, hsa-miR-1305, and hsa-miR-1306. Nevertheless, the expression of hsa-miR-146b attracted our attention the most as the statistical analysis showed that this miRNA was very significantly overexpressed in treated cells compared to untreated ones.

Next, we analysed the 12 sequences of potential miRNAs from *B2* RNA by qRT-PCR in synchronised cells treated or not with A190 (Table 2). Only four sequences were expressed in the NSCLC-N6 cells (Fig. 2). Of these four, only one sequence exhibited a significant overexpression in treated cells compared to untreated ones according to the statistical analysis of the results. Taken together, these findings confirm that the NSCLC-N6 cellular model expresses miRNAs.

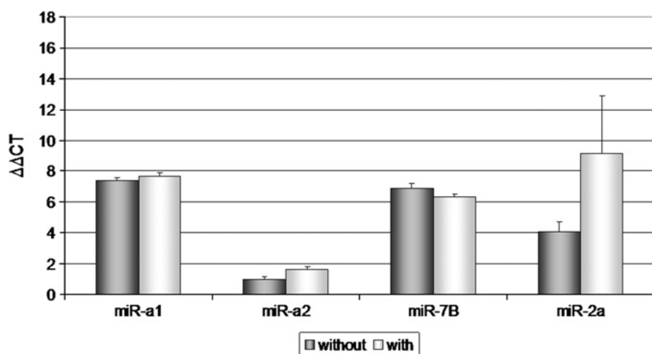

Fig. 2. The expression of different potential miRNAs designed by bioinformatic programme is tested by qRT-PCR on NSCLC-N6 cells treated or not with 39.4 μM of A190 for 60 h. The results are expressed as a ratio of mRNA quantity of the miRNA tested and that of β-actin (control gene).

We chose to analyse hsa-miR-146b due to its homology with exon 4 of the *HEF1* gene. The overexpression of hsa-miR-146b in synchronised cells treated with A190 for 60 h appeared to be very interesting. In fact, in view of its involvement in glioma and thyroid cancer (Nikiforova et al., 2008) and its overexpression in treated NSCLC-N6 cells, it could be implicated not only in the regulation of the *HEF1* gene at exon 4 but also, like *HEF1*, in apoptosis. We can therefore put forward two hypotheses about its origin. The first is that this miRNA, which targets *HEF1* at exon 4, could originate from chromosome 6 and, more particularly, the non-coding *B2* RNA. Indeed, the literature on large nc RNAs shows that they often have a regulatory function. Sometimes they are even precursors of miRNA such as the non-coding RNA H19 (Cai and Cullen, 2007). Thus, given its size, its homology with *HEF1* and the overexpression of the two genes after 60 h of treatment with A190, it is possible that *B2* RNA may generate miRNAs, which could notably regulate the *HEF1* gene. The second hypothesis is that this miRNA comes from *HEF1* itself. Thus, this gene would be self-regulated by means of the miRNAs. Under both these hypotheses, the regulation of the *HEF1* gene would be explained by the thresholds demonstrated by Dacke but not yet defined. In fact, the team of Dadke has demonstrated that *HEF1* acts on cell proliferation through the protein $p55^{hef1}$. However, when its expression is below a certain threshold, cell apoptosis is induced. Thus, expression of miRNAs would enable the quantity of mRNA of the *HEF1* gene to be reduced so falling below the threshold, inducing cell apoptosis. With the aim of testing our two hypotheses, we have amplified the precursor hsa-miR-146b and are determining its sequence in order to show whether it comes from *B2* RNA or the *HEF1* gene.

In addition, the qRT-PCR on the sequence miRNA 2A, which is a potential miRNA, showed a statistically significant overexpression in treated cells, like hsa-miR-146b. This sequence presents a homology with exon 2 of the *HEF1* gene. We suggest that it is a mature miRNA originating from *B2* or *HEF1* like hsa-miR-146b. It would be interesting to amplify and sequence its precursor in order to answer these questions.

5. Conclusion

In conclusion, this work has demonstrated the expression of miRNA in our NSCLC-N6 cellular model and, in particular, has shown the overexpression of hsa-miR-146b and a sequence miR-2a which could be a mature miRNA. This strategy of experiment has ever been done to characterise miRNAs such as biomarkers in the serum of renal cell carcinoma (Redova et al., n.d.). By this study, we show that the miRNAs overexpressed might be obtained from the structure of B2 and might interact with HEF1. But in order to prove that we have to, in first time, sequence the hsa-miR-146b. Its sequencing reveals if they have any involvement in NSCLC and in cell proliferation *in vitro*. It will also reveal whether they originate from the *HEF1* or the *B2* gene. If they are from *HEF1*, we will have to confirm that this gene is self-regulated *via* miRNAs. In order to prove the regulation of *HEF1* by miRNAs of B2, we would downregulate the miRNA concerned and observe the impact on *HEF1* expression (Hamada et al., 2012). However, if they are from *B2*, this will confirm, on one hand, that non-coding *B2* RNA is definitely a precursor of miRNA and, on the other hand, that the *B2* gene is involved in the regulation of *HEF1 via* miRNAs.

Financial disclosure

This work was supported by grants from the University of Nantes and supported by Ligue Contre Le Cancer for a Ph.D. fellowship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

Cai, X., Cullen, B.R., 2007. The imprinted H19 noncoding RNA is a primary microRNA precursor. RNA 13, 313–316.
Calin, G.A., Croce, C.M., 2006. MicroRNA-cancer connection: the beginning of a new tale. Cancer Res. 66, 7390–7394.
Cimmino, A., et al., 2005. miR-15 and miR-16 induce apoptosis by targeting BCL2. Proc. Natl. Acad. Sci. U. S. A. 102, 13944–13949.
Esquela-Kerscher, A., Slack, F.J., 2006. Oncomirs — microRNAs with a role in cancer. Nat. Rev. Cancer 6, 259–269.
Giard, D.J., et al., 1973. *In vitro* cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J. Natl. Cancer Inst. 51, 1417–1423.
Greither, T., et al., 2010. Elevated expression of microRNAs 155, 203, 210 and 222 in pancreatic tumors is associated with poorer survival. Int. J. Cancer 126, 73–80.
Hamada, N., et al., 2012. MicroRNA expression profiling of NGF-treated PC12 cells revealed a critical role for miR-221 in neuronal differentiation. Neurochem Int.
Hayashita, Y., et al., 2005. A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. Cancer Res. 65, 9628–9632.
Jacquot, C., et al., 2004. Identification of a novel putative non-coding RNA involved in proliferation arrest of a non-small cell lung carcinoma cell line treated with an original chemical substance, methyl-4-methoxy-3-(3-methyl-2-butanoyl) benzoate. Int. J. Oncol. 25, 519–527.
Kotsakis, A., Georgoulias, V., 2010. Targeting epidermal growth factor receptor in the treatment of non-small-cell lung cancer. Expert. Opin. Pharmacother. 11 (14), 2363–2389.
Law, S.F., et al., 1996. Human enhancer of filamentation 1, a novel p130cas-like docking protein, associates with focal adhesion kinase and induces pseudohyphal growth in Saccharomyces cerevisiae. Mol. Cell. Biol. 16, 3327–3337.
Malleter, M., et al., 2010. A novel large regulator RNA, B2, partially overlaps the HEF1/NEDD9/Cas-L gene. Int. J. Mol. Med. 25, 897–903.
Moreau, D., et al., 2008. Original triazine inductor of new specific molecular targets, with antitumor activity against nonsmall cell lung cancer. Int. J. Cancer 123, 2676–2683.
Nikiforova, M.N., et al., 2008. MicroRNA expression profiling of thyroid tumors: biological significance and diagnostic utility. J. Clin. Endocrinol. Metab. 93, 1600–1608.
Niu, G., Chen, X., 2010. Vascular endothelial growth factor as an anti-angiogenic target for cancer therapy. Curr. Drug Targets 11, 1000–1017.
O'Neill, G.M., Golemis, E.A., 2001. Proteolysis of the docking protein HEF1 and implications for focal adhesion dynamics. Mol. Cell. Biol. 21, 5094–5108.
Redova, M., et al., Circulating miR-378 and miR-451 in serum are potential biomarkers for renal cell carcinoma. J Transl Med.10, 55.
Roussakis, C., et al., 1991. Study of *in vitro* drug sensitivity on a newly established cell line from a primary bronchial epidermoid carcinoma of human origin (NSCLCN6). Anticancer. Res. 11, 2239–2244.
Sakai, R., et al., 1994. A novel signaling molecule, p130, forms stable complexes *in vivo* with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner. EMBO J. 13, 3748–3756.
Sawin, K.E., Endow, S.A., 1993. Meiosis, mitosis and microtubule motors. BioEssays 15, 399–407.
Suzuki, K., Takahashi, K., 2003. Reduced cell adhesion during mitosis by threonine phosphorylation of beta1 integrin. J. Cell. Physiol. 197, 297–305.
Takamizawa, J., et al., 2004. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. Cancer Res. 64, 3753–3756.
Wong, K.K., 2009. Recent developments in anti-cancer agents targeting the Ras/Raf/MEK/ERK pathway. Recent Pat. Anticancer Drug Discov. 4, 28–35.
Xia, H., et al., 2009. microRNA-146b inhibits glioma cell migration and invasion by targeting MMPs. Brain Res 1269, 158–165.
Zhang, B., et al., 2007. microRNAs as oncogenes and tumor suppressors. Dev. Biol. 302, 1–12.
Zinner, R.G., Fossella, F.V., Herbst, R.S., 2004. Pemetrexed in advanced NSCLC: a review of the clinical data. Oncology (Williston Park) 18, 54–62.