



MiR-483-5p and miR-139-5p promote invasion and tumorigenicity of adrenocortical carcinoma cells

Claire Agosta

► To cite this version:

Claire Agosta. MiR-483-5p and miR-139-5p promote invasion and tumorigenicity of adrenocortical carcinoma cells. Human health and pathology. 2015. dumas-01518315

HAL Id: dumas-01518315

<https://dumas.ccsd.cnrs.fr/dumas-01518315>

Submitted on 4 May 2017

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



**UFR de
Médecine**

**UNIVERSITÉ
Grenoble
Alpes**



**BIBLIOTHÈQUES
UNIVERSITAIRES
Grenoble Alpes**



AVERTISSEMENT

Ce document est le fruit d'un long travail approuvé par le jury de soutenance et mis à disposition de l'ensemble de la communauté universitaire élargie.

Il n'a pas été réévalué depuis la date de soutenance.

Il est soumis à la propriété intellectuelle de l'auteur. Ceci implique une obligation de citation et de référencement lors de l'utilisation de ce document.

D'autre part, toute contrefaçon, plagiat, reproduction illicite encourt une poursuite pénale.

Contact au SID de Grenoble :
bump-theses@univ-grenoble-alpes.fr

LIENS

Code de la Propriété Intellectuelle. articles L 122. 4

Code de la Propriété Intellectuelle. articles L 335.2- L 335.10

<http://www.cfcopies.com/juridique/droit-auteur>

<http://www.culture.gouv.fr/culture/infos-pratiques/droits/protection.htm>

UNIVERSITE JOSEPH FOURIER
FACULTE DE MEDECINE DE GRENOBLE

Année : 2015

N°

MiR-483-5p et miR-139-5p

contribuent à l'invasivité et la tumorigénicité

du carcinome corticosurrénalien

**THÈSE - ARTICLE
PRÉSENTÉE POUR L'OBTENTION DU DOCTORAT EN MÉDECINE
DIPLÔME D'ÉTAT**

D.E.S d'ENDOCRINOLOGIE, DIABÈTE, MALADIES MÉTABOLIQUES

Claire AGOSTA

[Données à caractère personnel]

Thèse soutenue publiquement à la faculté de médecine de Grenoble*
Le 13 novembre 2015

DEVANT LE JURY COMPOSÉ DE :

Président du jury : M. le Professeur Olivier CHABRE

Directeur de thèse : Me. le Docteur Nadia CHERRADI

Membres du jury :

M. le Professeur Philippe CHAFFANJON

Me. le Professeur Pascale HOFFMANN

Me. le Docteur Nathalie STURM

**La Faculté de Médecine de Grenoble n'entend donner aucune approbation ni improbation aux opinions émises dans les thèses ; ces opinions sont considérées comme propres à leurs auteurs.*

REMERCIEMENTS

Tout d'abord, je remercie sincèrement les membres du jury d'avoir jugé mon travail de thèse,

A Monsieur le Professeur Olivier Chabre,

Merci de m'avoir formée et guidée pendant ces 5 et bientôt 6 années internat. Vous savez nous transmettre l'amour de la médecine et surtout la passion de l'endocrinologie ! Après un externat à Marseille, je suis très heureuse d'avoir choisi la montagne à Grenoble pour mon internat et de vous avoir eu comme maître. Vous êtes un modèle d'enseignement et vous m'avez donné des bases solides pour l'exercice de cette belle spécialité. Je vous remercie pour votre écoute et vos encouragements pendant mes études, ainsi que votre soutien pour allier vie personnelle et professionnelle.

A Madame le Docteur Nadia Cherradi,

Grâce à toi j'ai passé une merveilleuse année de recherche dans le laboratoire de biologie du cancer et de l'infection au CEA de Grenoble. J'y ai été si bien accueillie par toute l'équipe que je remercie chaleureusement (Jean Jacques Feige, Nadia A., Agnès, Fred, Mickael, Aude, Christine, Josiane, Sophie, Khadija, Waël, Féli, Vanessa...). Merci à Jonathan que je n'ai pas eu l'occasion de connaître mais qui a beaucoup travaillé sur les suites de ce projet et qui l'a enrichi. J'ai particulièrement apprécié travailler avec toi Nadia pour ta rigueur, ton encadrement, et ton enthousiasme au travail. Merci pour ce que tu m'as apporté sur le plan scientifique, grâce à toi la recherche fondamentale n'est (presque) plus un secret pour moi et je dois dire que j'y ai pris goût ! Merci aussi pour ton amitié qui je l'espère perdurera.

A Monsieur le Professeur Pierre-Yves Benhamou,

Comme je vous l'ai dit je regrette votre absence aujourd'hui comme membre du jury, mais vous savez mon estime pour vous. Je vous remercie pour vos qualités humaines que nous avons pu admirer en visite auprès des patients, et aussi auprès de nous les internes (qui n'a pas versé une larme à vos côtés ?). Votre expertise en diabétologie est pour moi une référence, et vos qualités de chercheur toujours connecté à la nouvelle technologie me fascine.

A Madame le Docteur (et Professeur) Nathalie Sturm,

Merci pour mon semestre en anatomopathologie qui a failli faire changer mon choix de spécialité. Tu m'as fait découvrir les lames de thyroïde, hypophyse et surrénale, décrites de façon précise par ton grand savoir faire. Nous sommes fiers de t'avoir comme référente de l'endocrino-pathologie. Merci pour ton aide pour la mise en place de la collection de tumeurs surrénales du CRB qui n'existerait pas sans ta rigueur dans la conservation des échantillons tumoraux depuis des années. Les couloirs du DAPC me sont devenus familiers et je remercie les correspondants du CRB qui m'ont permis d'avancer dans la banque : monsieur Philippe Lorimier et Gabriel. J'en profite pour dire mon amitié à Diane et Julie, internes et futures assistantes d'anapath, avec qui nous avons passé de belles années.

A Monsieur le Professeur Philippe Chaffanjon,

Vous me faites l'honneur de juger mon travail de recherche sur la carcinogénèse corticosurrénalienne, domaine qui vous est cher, je vous en remercie. La collaboration entre la chirurgie, l'endocrinologie clinique, l'anatomo-pathologie et la recherche fondamentale est primordiale pour la prise en charge des patients.

A Madame le Professeur Pascale Hoffmann,

Tout d'abord merci pour votre accueil dans votre service de PMA, j'y ai été très bien formée et ce semestre a confirmé mon choix d'exercice professionnel en alliant la médecine de la reproduction et l'endocrinologie. J'apprécie la place que vous donnez aux endocrinologues dans votre service et notamment aux internes en formation. Je vous remercie de juger mon travail aujourd'hui, car en plus de votre qualité de gynécologue-obstétricienne, j'ai pu connaître votre ouverture d'esprit pour bien d'autres domaines cliniques et de recherche fondamentale. En effet, vous faites partie de la même équipe de recherche sur l'Angiogenèse physiologique et tumorale dirigée par Jean-Jacques Feige au CEA de Grenoble, dans laquelle a été réalisé ce travail.

~~~~~

Je remercie aussi l'équipe de PMA adorable avec moi ces 6 derniers mois juste avant la thèse... En particulier Nicole, Agnès, Sylvie, Monique, Brigitte, Eliane, Odile, Aurore, Laure et Alexia. Vous avez été d'un grand soutien et m'avez permis d'affronter un épisode difficile dont on se souviendra de très loin... Je me suis sentie dans une grande famille. Je remercie tout particulièrement Nicole qui m'a beaucoup appris de cette belle spécialité et auprès de qui on apprécie tous travailler de par son

expérience médicale et ses grandes qualités humaines ! Bravo pour cette belle équipe de PMA du CHU de Grenoble (biologistes, médecins, sages femmes, secrétaires...), pour votre rôle majeur auprès des couples et pour avoir renforcé mon goût pour la médecine de la reproduction.

~~~~~

Merci à toute l'équipe d'endocrinologie et de diabétologie du CHU de Grenoble. J'ai passé des belles et longues années d'internat dans le pavillon des ECRINS dont je connais les recoins mais qui ne cesse d'évoluer.

Merci aux médecins de l'équipe. J'apprécie les relations bienveillantes et les compétences de chacun, complémentaires et riches. Merci à Nelly, Marie, Anne-Laure B., Sandrine L., Isabelle, Sandrine C. et aussi à mes merveilleuses assistantes Marine C., Marine B. et Marie Aude !

Un message nostalgique pour les infirmières, aides-soignantes et secrétaires du service d'endocrinologie avec qui j'ai passé 1 an en tant qu'interne au deuxième étage... Ces deux semestres ont été riches en émotions et j'ai vraiment apprécié y travailler pour un intérêt commun ! Hélène (la doyenne), Coco, Blandine, Dominique, Lydia, Marie-Agnès, Marie-Odile, Béa, Ratiba, Marie-O, Gaëlle, Murielle, Marie-Antoinette, Sylvie...

Last but not least, merci à la dream-team des co-internes d'endoc-diabéto, toujours très soudée (c'est une chance !) et avec qui on passe des moments inoubliables dans le service et ailleurs (je pense aux mojitos géants du bar du cousin à Sandra aux JNDES annuelles à Paris...on s'en souvient !!). Je suis persuadée que notre amitié perdurera en plus de notre lien en tant que collègues.

Maurine, avec qui j'ai passé un premier semestre aux ECRINS mémorable accompagnées des zouzous Benoit ,Jojo et Georgounet ! et ça continue... Bonne suite pour toi à Chambé avec Cyril et futur baby... que du bonheur.

Sandra et Marie, on va finir l'internat quasi en même temps, et je m'en réjouie. J'apprécie l'intimité et la confiance dans nos relations, et qui ne se perdent pas ☺.

Marie-Aude, Anne Laure C. et Claudine, quelle joie de vous connaître et de partager l'amour de la « marmite » ☺.

Enfin un message pour l'équipe de diabétologie de la Martinique, le Dr Fagour notamment qui m'a accueillie dans son service. J'ai passé un semestre rêvé d'inter-CHU très enrichissant.

~~~~~

Il me reste à remercier mes proches sans qui la vie professionnelle n'a aucun sens !

Claire, mon amie de toujours et qui partage la même passion de l'endocrinologie. Je te remercie pour ton soutien, ton écoute et tes grandes qualités. Nos chemins sont liés ☺, tout comme avec Milie (un rayon de soleil) vous comptez beaucoup pour moi !

Mica, Rej, Quentin, Marianne... les années folles du lycée Thiers, puis chacun a pris un horizon différent... mais pour toujours mieux se retrouver !

Mes copains d'enfance de Martinique, bien entendu Olivia et Michou, et les Beauvais, Anton, Oliviers... la belle époque qui continue malgré la distance !! je vous porte dans mon cœur.

A mon parrain et ma marraine, tous mes oncles et tantes, mes cousins si nombreux avec qui je garde des liens intenses. Clin d'œil à Caro la pionnière des Grenoblois !

A mes grands parents chéris, mémé de Carry et mon pépé, mamie et bon papa : je vous aime !

A ma belle famille avec qui je vis de bons moments et où je me sens bien.

Cécile, Cyril et Lucie, mes frérots adorés, toujours soudés... vous êtes tout pour moi ! Et longue vie à la marmaille actuelle (Gwénaël, Alizé, Timéo) et future... Thomas et Cécile merci pour votre aide grâce à votre côté geek bien pratique quand il y a alerte rouge (ou un choix de pomme à faire) ☺.

A mes parents adorés, vous m'avez toujours guidée et soutenue, votre présence m'est indispensable. Ma vocation de médecin n'a pas été simple tous les jours parce qu'elle « consomme » beaucoup de temps... Heureusement, à vos côtés j'ai appris que l'épanouissement professionnel est une chose, mais que la richesse de la vie personnelle est plus important que tout (vive les vacances en famille).

Enfin, à mes deux (bientôt trois et pourquoi pas quatre) amours de ma vie.... Mon doudou, Maël et mon choubidou, Timéo. Quel boulot cette fin d'année 2015 avec nos deux thèses ! ouf... un peu (ou beaucoup) de répit nous attend ☺. Merci pour tous les bons moments, et la traversée des plus difficiles... ce qui montre bien que notre projet de vie est plus fort !! Love.

## TABLE DES MATIÈRES

|                                                                                                                 |           |
|-----------------------------------------------------------------------------------------------------------------|-----------|
| <b>Remerciements</b>                                                                                            | <b>2</b>  |
| <b>Table des matières</b>                                                                                       | <b>6</b>  |
| <b>Résumé</b>                                                                                                   | <b>8</b>  |
| <b>Abstract</b>                                                                                                 | <b>9</b>  |
| <b>MiR-483-5p and miR-139-5p promote invasion and tumorigenicity of adrenocortical carcinoma cells</b>          | <b>10</b> |
| <i>INTRODUCTION</i>                                                                                             | 11        |
| <i>METHODS</i>                                                                                                  | 14        |
| Patient cohort                                                                                                  | 14        |
| Cell culture                                                                                                    | 14        |
| NCI H295R cells transfection                                                                                    | 15        |
| Total RNA extraction and quantification of microRNAs using RT-qPCR                                              | 15        |
| Prediction of miRNA target genes                                                                                | 16        |
| Quantification of target mRNA levels by real time RT-qPCR                                                       | 17        |
| Analysis of protein expression by Western blot                                                                  | 18        |
| Luciferase reporter gene assay                                                                                  | 19        |
| Cell proliferation analysis                                                                                     | 20        |
| Cell cycle and apoptosis analysis                                                                               | 20        |
| Transwell migration and invasion assay                                                                          | 21        |
| Anchorage-independent colony formation assay in soft agar                                                       | 22        |
| Statistical analyses                                                                                            | 22        |
| <i>RESULTS</i>                                                                                                  | 23        |
| miRNAs expression profile in tumor tissues and identification of putative target genes                          | 23        |
| Inverse correlation between NDRG2/NDRG4 expression and miR-483-5p/miR-139-5p expression levels in tumor tissues | 24        |
| Effect of miR-483-5p and miR-139-5p inhibitors on NDRG expression in NCI H295R cells                            | 24        |

|                                                                                                                                                               |    |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| MiR-483-5p and miR-139-5p functionally interact with the 3'-untranslated region of NDRG2 and NDRG4, respectively                                              | 25 |
| MiR-483-5p and miR-139-5p did not affect NCI H295R cell proliferation or apoptosis.                                                                           | 26 |
| MiR-483-5p and miR-139-5p promote migration and invasion of NCI H295R cells                                                                                   | 27 |
| MiR-483-5p and miR-139-5p are involved in anchorage-independent colony formation of NCI H295R cells                                                           | 27 |
| <i>DISCUSSION</i>                                                                                                                                             | 29 |
| <b>Références</b>                                                                                                                                             | 35 |
| <b>Table and Figures</b>                                                                                                                                      | 41 |
| Table 1. Putative miRNA/mRNA target interaction in ACC.                                                                                                       | 41 |
| Figure 1. Schematic representations of miR-483-5p and miR-139-5p interaction with NDRG2 and NDRG4 respectively, according to prediction softwares.            | 41 |
| Figure 2. Inverse correlation between microRNAs expression and putative target gene expression in a cohort of human adrenocortical tissues.                   | 42 |
| Figure 3. Effect of miR-483-5p and miR-139-5p inhibitors on NDRG expression in NCI H295R cells.                                                               | 42 |
| Figure 4. MiR-483-5p and miR-139-5p functionally interact with NDRG2 and NDRG4 3'UTR.                                                                         | 43 |
| Figure 5. Effect of miR-483-5p and miR-139-5p inhibition, or miR-195 and miR-335 overexpression, on cell cycle and cell proliferation in NCI-H295R cell line. | 44 |
| Figure 6. Effect of miR-483-5p and miR-139-5p inhibition, or miR-195 and miR-335 overexpression, on NCI-H295R cell apoptosis.                                 | 44 |
| Figure 7. Effect of miR-483-5p and miR-139-5p inhibition, or NDRG target gene overexpression on migration and invasion capacities of NCI-H295R cells.         | 45 |
| Figure 8. Effect of miR-483-5p and miR-139-5p inhibition on anchorage-independent growth of NCI-H295R cells.                                                  | 45 |
| Figure 9. The multiple functions of the N-Myc Downstream-Regulated gene (NDRG) family.                                                                        | 46 |
| <i>CONCLUSION</i>                                                                                                                                             | 57 |
| <b>Intégration de la collection de tumeurs surrénales dans le centre de ressources biologiques (CRB) du CHU de Grenoble</b>                                   | 59 |
| <b>Liste des enseignants à l'UFR de médecine</b>                                                                                                              | 63 |
| <b>Serment d'hippocrate</b>                                                                                                                                   | 67 |

## RÉSUMÉ

**Contexte.** Les microARNs ont émergé comme bio-marqueurs potentiels pour le diagnostic et le pronostic des tumeurs corticosurrénaliennes (TCS). Notre équipe a montré que miR-483-5p et miR-139-5p sont sur-exprimés dans les carcinomes corticosurrénaliens (CCS) (Chabre et al. 2013). Toutefois, le rôle de ces miRNAs dans la pathogenèse des CCS est encore inconnu.

**Objectif.** L'objectif était d'identifier les gènes cibles de miR-483-5p et miR-139-5p dans le CCS et d'étudier leur contribution dans la tumorigenèse corticosurrénalienne.

**Méthodes.** La prédiction des cibles a été réalisée en utilisant les bases de données miRWalk et TargetScan, combinées avec le profil d'expression génique des tumeurs corticosurrénaliennes, publié précédemment (de Reyniès et al. 2009). Le niveau d'expression tumoral de miR-483-5p et miR-139-5p a été mesuré par RT-qPCR dans une cohorte de 28 patients issus de la banque du réseau national COMETE (COrtico- MEdullo Tumeurs Endocrines), et corrélé au niveau d'expression des gènes cibles hypothétiques. La région 3'-non traduite (3'UTR) des gènes cibles a été clonée en aval d'un gène rapporteur Luciferase pour évaluer l'interaction microARN-ARN messager. Le rôle des deux microARNs dans le phénotype malin de la lignée cellulaire NCI- H295R a été étudié par transfection d'inhibiteurs de microARNs.

**Résultats.** Nous avons identifié NDRG2 (N-Myc Downstream-Regulated Gene 2) et NDRG4 comme cibles de miR-483-5p et miR-139-5p, respectivement. L'expression de NDRG2 et NDRG4 dans les CCS était inversement corrélée à l'expression de miR-483-5p et miR-139-5p, respectivement. L'inhibition de miR-483-5p et miR-139-5p dans les cellules NCI-H295R entraîne une augmentation du niveau d'ARNm de NDRG2 et NDRG4. De plus, les transcrits de NDRG2 et NDRG4 sont régulés négativement via un site d'interaction spécifique avec les microARNs. Enfin, l'inhibition de miR-483-5p et de miR-139-5p n'a pas affecté la prolifération ou l'apoptose des cellules NCI- H295R, mais a inhibé leur caractère invasif et leur croissance en gel d'agar sans ancrage cellulaire.

**Conclusion.** Nos données suggèrent que miR-483-5p et miR-139-5p contribuent à l'agressivité des CCS en réprimant l'expression de deux gènes suppresseurs de tumeurs, NDRG2 et NDRG4.

**Mots clefs.** microARNs, carcinome corticosurrénalien, N-Myc Downstream-Regulated Genes, invasion, miR-483-5p, miR-139-5p

## ABSTRACT

**Background.** MicroRNAs have emerged as potential biomarkers for diagnosis and prognosis of adrenocortical tumors. We have previously shown that miR-483-5p and miR-139-5p were up-regulated in adrenocortical carcinomas (ACC) (Chabre et al. 2013). However, the role of these microRNAs in the pathogenesis of ACC is still unknown.

**Objective.** Our aim was to identify target genes of miR-483-5p and miR-139-5p in ACC and to investigate their contribution to adrenocortical tumorigenesis.

**Methods.** Target prediction was performed using miRWALK and TargetScan databases combined with the previously published transcriptome of adrenocortical tumors (de Reyniès *et al.* 2009). Tumor miR-483-5p and miR-139-5p levels were measured by RT-qPCR in 28 patients from the French biobank COMETE and correlated with the expression of their putative targets. The 3'-untranslated regions of putative target genes were cloned into pMiR-Luciferase vectors to evaluate miRNA-mRNA interactions. The role of miR-483-5p or miR-139-5p in the malignant phenotype of NCI-H295R cells was investigated using miRNA inhibitors.

**Results.** We identified NDRG2 (N-Myc Downstream-Regulated Gene 2) and NDRG4 as targets of miR-483-5p and miR-139-5p, respectively. In ACC, expression of NDRG2 and NDRG4 was inversely correlated with miR-483-5p and miR-139-5p levels. Downregulation of miR-483-5p or miR-139-5p in NCI-H295R cells induced an increase of NDRG2 or NDRG4 mRNAs. Moreover, NDRG2 and NDRG4 transcripts were negatively regulated via specific miRNA target sites. Finally, silencing miR-483-5p or miR-139-5p did not affect proliferation or apoptosis of NCI-H295R cells but inhibited their anchorage-independent growth and invasion capacities.

**Conclusion.** Our data indicate for the first time that repression of NDRG2 and NDRG4 tumor suppressor genes by overexpressed miR-483-5p and miR-139-5p might contribute to ACC aggressiveness.

**Keywords.** microRNAs, adrenocortical carcinoma, N-Myc Downstream-Regulated Genes, invasion, miR-483-5p, miR-139-5p

# **MiR-483-5p and miR-139-5p promote invasion and tumorigenicity of adrenocortical carcinoma cells**

C. Agosta<sup>1,2</sup>, O. Chabre<sup>2,3,4,5</sup>, JJ. Feige<sup>3,4,5</sup> and N. Cherradi<sup>3,4,5</sup>

<sup>1</sup>Joseph Fourier University, UFR Pharmacie, F-38706 La Tronche, France

<sup>2</sup>Hospital Albert Michallon, Departement of Endocrinology, F-38054 Grenoble, France

<sup>3</sup>Institut National de la Santé et de la Recherche Médicale, Unité 1036, F-38054 Grenoble, France

<sup>4</sup>Commissariat à l'Energie Atomique, Institute of Life Sciences Research and Technologies, Biology of Cancer and Infection, F-38054 Grenoble, France

<sup>5</sup>Université Grenoble Alpes, Unité Mixte de Recherche-S1036, 38000, Grenoble, France

**Correspondence to:** [nadia.cherradi@cea.fr](mailto:nadia.cherradi@cea.fr)

**Short title:** miR-483-5p and miR-139-5p in adrenocortical carcinoma

**Word count:** abstract 262 words, text 6570 words

**Number of references:** 66

**Number of tables and figures:** 1 Table, 9 Figures

## **List of abbreviations**

aACC: aggressive AdrenoCortical Carcinoma

naACC: non aggressive AdrenoCortical Carcinoma

ACA: AdrenoCortical Adenoma

IGF: Insulin-like Growth Factor

NDRG: N-Myc Downstream-Regulated Genes

PCR: Polymerase Chain Reaction

RNA: Ribonucleic Acid

UTR: Untranslated Region

## INTRODUCTION

Adrenocortical carcinoma (ACC) is a rare cancer with an incidence of 0.7–2 cases per million people per year. Nevertheless, ACC is a highly aggressive tumor with very poor prognosis. ACC can occur at any age, with a peak incidence between 40 and 50 years (1). The poor prognosis is attributable in part to the fact that many ACCs are not detected until they are at advanced stage: 36% to 46.6% of patients are stage IV at the time of diagnosis, as defined by the presence of distant metastasis according to the European Network for the Study of Adrenal Tumors (ENS@T) classification in two independent large cohorts of the German adrenocortical carcinoma registry and the U.S. National Cancer Institute SEER (Surveillance, Epidemiology and End Results) database, respectively (2,3). The 5-year disease-specific survival rate for stage IV patients in those cohorts was 13% and 6.9% respectively, and around 38.1% in the overall population (ACC stage I to IV). The only curative treatment for localized ACC (ENS@T stage I, II and III) is radical surgery, but postoperative recurrence is frequent. Unfortunately, treatment for aggressive form of ACC is limited and still ineffective (4–7). The tumor-node–metastasis (TNM) classification proposed by ENS@T in 2008 has shown a higher prognostic potential, and is now used as the reference staging system of ACC tumors (2). Other prognosis factors for recurrence used in clinical practice are resection status after surgery and proliferation markers including mitotic count and Ki-67 index in the primary tumor (8).

Therefore, there is a need for a better characterization of the genetic alterations and molecular mechanisms involved in sporadic tumor development in order to improve diagnosis and therapeutic medical care of those patients (5,9–12). Over the last decade, novel approaches have been used to identify cancer signatures by using high-throughput technologies for pan-genomic analysis of genetic and epigenetic alterations as well as gene expression profiling of ACC tumors. The integration of the 'omics' using tumor genomic (DNA mutations and chromosomal aberrations), transcriptomic (mRNA

and microRNA expression patterns) and epigenomic (DNA methylation profiles) data improve the diagnosis and prognosis classification of patients with ACC into subgroups with different clinical outcome, (13). The alteration mostly observed in sporadic ACCs is overexpression of IGF-2 growth factor, in almost 90% of ACC tumors. This alteration has been however observed also in rare cases of adrenocortical adenomas (ACAs) (14,15). Furthermore, other molecular pathways playing a major role in ACC pathogenesis are frequently altered by somatic gene mutations detected in two third of ACC tumors with poor outcome. The most frequent mutations are found in genes acting in the Wnt/Beta-catenin pathway and the TP53 signaling pathway or in cell cycle control (10,16–19). Nonetheless, these alterations do not concern all ACCs thus suggesting that other unidentified molecular defects are involved in the pathogenesis of ACC. Notably, alterations in microRNA expression profile is another interesting pathway, which is investigated more and more in adrenocortical tumors. Among others, miR-483-5p was found by different research groups to be the most significantly overexpressed microRNA in ACC tissue (20–27). More recently, we were first to show that miR-483-5p was increased in the serum of ACC patients (28). Further work confirmed our observations (29,30). Importantly, our data suggest that circulating miR-483-5p could be used as a biomarker for aggressive ACC (28).

MicroRNAs are small non-coding RNAs that regulate the expression of more than 50% coding genes at the post-transcriptional level through their binding to the 3'-untranslated region (UTR) of the target gene messenger RNA (mRNA). They are involved in a wide range of key biological mechanisms such as cell-cycle control, apoptosis, proliferation, differentiation, migration and invasion. MicroRNAs cause mRNA degradation or translational repression by perfect or imperfect pairing with the 3'UTR region of mRNAs, respectively (31). A large number of recent studies have shown that deregulation of microRNA expression is associated with a variety of tumors. These microRNAs play a tumor suppressor or oncogenic role depending of the cellular context (32). They exert their effect in

a tissue-specific manner depending on cancer type but also on tumor stage. In adrenocortical tumors, miR-195 and miR-335 were found down-regulated whereas miR-483-5p was upregulated in ACCs compared with ACAs. In addition, miR-139-5p was overexpressed only in aggressive ACC (recurring or metastatic tumors) but not in recurrence-free ACCs (28).

Nevertheless, up to now, the functional role of these microRNAs in ACCs has not been explored in depth. The pathways affected by differentially expressed microRNAs could represent potent targets for anti-cancer therapy and personalized medicine. Clinical applications of direct or indirect microRNA targeting for adrenal tumors need better characterization of the pathways altered by microRNAs and leading to ACC tumorigenesis.

In this study, using an aggressive human ACC cell line, we investigated the functional role of miR-483-5p and miR-139-5p at the molecular level by identifying their potential target genes and by analyzing their impact on basic cellular processes.

## *METHODS*

### **Patient cohort**

The adrenocortical tumor samples were obtained from a validation cohort of 28 patients that has been described in a recent study (28). Ten adrenocortical adenomas (ACA) and 18 ACC were collected from the national tumor bank of the Cortico-Medulo Tumeurs Endocrines (COMETE) network (Cochin Hospital, Paris). Adrenocortical carcinoma (ACC) samples were divided into two groups: a first group consisting of aggressive tumors (aACC, n=9) and a second group consisting of non-aggressive tumors (naACC, n=9). Three normal adrenal cortex tissues (NA) were obtained from nephrectomized patients.

### **Cell culture**

The human adrenocortical carcinoma NCI-H295R cell line was a gift from Dr William E. Rainey (Department of Molecular and Integrative Biology, University of Michigan). Cells were cultured as a monolayer on rat tail Collagen I-coated plates (0.07 mg/ml, BD Biosciences) in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12; GIBCO Life Technologies) supplemented with 1% (v/v) ITS Premix Universal Culture Supplement (containing insulin, transferrin, selenium, bovine serum albumin and linoleic acid, Corning Discovery Labware), 5% (v/v) Cosmic Calf Serum (HyClone, Thermo Scientific) and antibiotics (1:100 Penicillin-Streptomycin and 1:1000 Gentamycin, GIBCO Life Technologies). HEK293 cells, derived from human embryonic kidney, were purchased from ATCC and cultured in DMEM GlutaMAX High Glucose medium (Invitrogen) containing 10 % of fetal bovine serum (GE Healthcare) and antibiotics. Cells were cultured in a humidified incubator at 37°C in a 5% CO<sub>2</sub>-95% air atmosphere.

## **NCI H295R cells transfection**

The NCI H295R cells were transiently transfected using commercially available mirVANA miRNA inhibitors (Ambion, Life Technologies) designed to inhibit the activity of endogenous mature miRNAs when introduced into cells. 50 nM of mirVANA miRNA inhibitors against hsa-miR-483-5p (Assay ID: MH11749) and hsa-miR-139-5p (Assay ID: MH12629) were transfected using 5 µl per well (6-well cell culture plate) or 2 µl per well (12-well plate) of Lipofectamine RNAiMAX (Invitrogen, Life Technologies) according to the manufacturer's instructions. Briefly, the day before transfection cells were seeded in antibiotic-free medium until 50% confluence. Opti-MEM I Reduced Serum Medium (Invitrogen, Life Technologies) was used to dilute microRNAs and Lipofectamine. Alternatively, NCI H295R cells were transiently transfected with mirVANA miRNA mimics at a final concentration of 50 nM (Assay ID: hsa-miR-195: MC10827; hsa-miR-335: MC10063) using the same transfection reagent. For mock transfection conditions, Lipofectamine RNAiMAX was used in the absence of mimics or inhibitors. mirVANA miRNA Negative Control (Ambion, Life Technologies) was transfected as a non-specific random sequence miRNA validated to not produce measurable effects on known miRNA function. The efficiency of miRNA over-expression or miRNA knockdown was assessed by reverse transcription (RT) and quantitative PCR (RT-qPCR) 24h, 48h, and 72h after transfection.

## **Total RNA extraction and quantification of microRNAs using RT-qPCR**

Total RNA as well as total protein extracts from NCI H295R cells were isolated simultaneously from the same sample using mirVANA PARIS Kit (Ambion, Life Technologies) according to the manufacturer's instructions. MiRNA levels in cell lysates were measured by RT-qPCR using TaqMan miRNA assays (Life Technologies). Ten nanograms of cell total RNA were reverse-transcribed using the TaqMan miRNA Reverse Transcription kit and miRNA-specific stem-loop primers (Applied

Biosystems, Life Technologies) in a 15 µl-RT reaction (composed of 0.15 µl 100 mM dNTPs mix, 1.5 µl 10x RT buffer, 0.19 µl RNase inhibitor (20 units/µl), 4.16 µl H<sub>2</sub>O, 1 µl multiscribe reverse transcriptase, and 5 µl input RNA), using a TGradient Thermal Cycler (Biometra, Goettingen, Germany) at 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. Real time PCR was performed on the 5'-extended cDNA with Applied Biosystems TaqMan 2X Universal PCR Master Mix and the appropriate 5'-TaqMan MicroRNA Assay Mix for each miRNA of interest (Assay ID: hsa-miR-483-5p: 002338; hsa-miR-139-5p: 002289; hsa-miR-195: 000494; hsa-miR-335: 000546 and RNU48: 001006). Briefly, 4.5 µl of 2.5-fold diluted RT product was combined with 5.5 µl of PCR assay reagents to generate a PCR volume of 10 µl. Real-time PCR was carried out on a C1000 Thermal cycler (CFX96 Real Time system, Bio-Rad) at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Data were analyzed with CFX Manager Software version V1.5.534.0511 (Bio-Rad). All experiments were performed duplicate. The RNU48 RNA was used as an endogenous control for normalization in NCI H295R cells. Normalized expression was calculated using the comparative Ct method and fold changes were derived from the 2<sup>-ΔΔCt</sup> values for each miRNA.

### Prediction of miRNA target genes

The target genes of the deregulated microRNAs were predicted using different algorithms based on complementary approaches (33): TargetScan which is based on sequence complementarity (<http://www.targetscan.org>), RNAHybrid which is based on thermodynamic stability (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid>) and miRWalk which is based on comparative analysis of several softwares (<http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/index.html>). We selected target genes with the lowest p value (p<0,05) which were provided by these target prediction softwares and that had an inversely correlated expression level with the corresponding microRNA

expression levels (analysis of the previously published transcriptomic study of adrenocortical tumors by de Reynies et al (34)).

### **Quantification of target mRNA levels by real time RT-qPCR**

N-Myc Downstream-Regulated Gene 2 and 4 (NDRG2 and NDRG4) expression was assessed by real time RT-qPCR. A first step of reverse transcription was carried out with 1µg of total RNA extract from cell lysates or from tissue sample using iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions. Quantitative PCR was performed using 2.5 µl of 2.5-fold diluted RT product and 7.5 µl of reaction mix (composed of 5 µl of GoTaq qPCR Master Mix (Promega), 2.15 µl of H<sub>2</sub>O, and 0.35 µl of forward and reverse PCR Primers mixed together at a final primer concentration of 0.35 µM) to generate a PCR volume of 10 µl. All primers were purchased from Sigma-Aldrich (human RPL13A: forward primer 5'-TTAACCTCATGCGTTGCC-3' and reverse primer 5'-TTCCTTGCTCCCAGCTCCTATGT-3'; human HPRT: forward primer 5'-ATGGACAGGACTGAACGTCTTGCT-3' and reverse primer 5'-TTGAGCACACAGAGGGCTACAATG-3'; human NDRG2: forward primer 5'-GCTCTGTCACTTCACTGTCTAT-3' and reverse primer 5'-AGTGGCTGGAAGCAAGATTAA-3'; human NDRG4: forward primer 5'-CCGGCCTAACTAGCACTTAC-3' and reverse primer 5'-GTTCACACGTTCCAATCT -3'). Real-time PCR was carried out at 95°C for 2 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The expression of NDRG2 and NDRG4 were normalized to RPL13A and HPRT as housekeeping genes. Normalized expression was calculated as described above. Correlation between microRNAs and putative target gene expression level in tumor samples was performed using raw RT-qPCR expression data normalized with housekeeping gene expression in each sample.

## **Analysis of protein expression by Western blot**

Proteins were extracted from NCI H295R cells transfected with premiR-negative control, miR-483-5p or miR-139-5p inhibitors using Cell Disruption Buffer (Ambion, Life Technologies) and were quantitated with microBCA protein Assay kit (Thermo Scientific). SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (35). Total proteins extracts were solubilized in sample buffer (60 mM Tris-HCl, pH 6.8, 2 % SDS, 5 % β-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue), boiled for 5 min and loaded onto a 12% SDS-PAGE minigel. Electrophoresis was performed at 100 V for 30 min and afterwards at 150 V for 1h in a buffer containing 50 mM of Tris-HCl pH 8.6, 384 mM of Glycine and 0.1% (w/v) of SDS. SDS-PAGE-resolved proteins were electrophoretically transferred onto a polyvinylidene fluoride (PVDF) membrane as previously described (36). Following transfer, the membrane was incubated in a blocking buffer (Tris-buffer saline (TBS) containing 0.1 % Tween 20 and 5 % non-fat dry milk) for 1 h at room temperature. The blots were probed with monoclonal mouse anti-Actin (1:5000, Sigma-Aldrich), rabbit anti-NDRG2 or rabbit anti-NDRG4 (1:1000, Cell Signaling Technology) overnight at 4 °C in TBS/Tween. The membrane was thoroughly washed with the same buffer (3 x 10 min), then incubated for 1 hour with either horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5000, Thermo Fisher) or HRP-conjugated goat anti-rabbit IgG (1:3000, Thermo Fisher). The PVDF sheet was washed as above and the antigen-antibody complex revealed by Enhanced Chemiluminescence, using the detection kit from BioRad and BioMax Kodak films (Sigma-Aldrich). Quantification of the signals was performed using ImageJ software. Protein levels were normalized to Actin to compensate for protein loading variations. Values are presented relative to protein content of control samples.

## Luciferase reporter gene assay

The 3'UTR of NDRG2 and NDRG4 mRNA containing the predicted miR-483-5p and miR-139-5p target sequences, respectively, were amplified from cDNA extract of healthy human trophoblasts with high fidelity Advantage Genomic LA Polymerase (Sigma-Aldrich). Primers used for amplification were designed by adding a restriction enzyme site when necessary. The PCR products (708bp for NDRG2-3'UTR and 1158bp for NDRG4-3'UTR) were cloned into the pMIR-Report luciferase vector (Promega, Madison, WI) using the Multi-Cloning Site, downstream of the Firefly luciferase reporter gene and between Sac-I and Spe-I for NDRG2-3'UTR or between Sac-I and Hind-III for NDRG2-3'UTR. All the plasmids were fully sequenced. Nucleotide-substitution mutations were carried out using site-directed mutagenesis at the 3'UTR regions of NDRG2 and NDRG4 (QuickChange XL site-directed mutagenesis kit, Agilent Technologies) with specific primers (pMiR-Luc-3'UTR-Mutant-NDRG2 : forward primer 5'-TGGAGGGTGCCCTGGGCAGCCCCACAACCTCTGC-3' and reverse primer 5'-GCAGAAGTTGTGGGCTGCCAGGGCACCCCTCCA-3' ; pMiR-Luc-3'UTR-Mutant-NDRG4 : forward primer 5'- TTCATCCCTCTTGCAGCCCTAACACTGCAAA-3' and reverse primer 5'-TTTGCAGTGTAGGCAGCGCAAGAGGGATGAA-3') and the template corresponding to wild-type 3'UTRs (**Figure 1**). Post-transcriptional inhibition of the luciferase reporter gene by miR-483-5p and miR-139-5p was assayed in HEK 293 cells. Briefly,  $1.5 \times 10^5$  cells were seeded in duplicates into 12-well plates one day before the transfection and then co-transfected with either miR-483-5p mimics, miR-139-5p mimics or miRNA negative control at a final concentration of 50 nM, together with 1ng of pMIR-Luc Report construct containing NDRG2-3'UTR, NDRG4-3'UTR or Mutant-3'UTR, and with 50 ng of pRL-Tk-Renilla luciferase plasmid. Lipofectamine 2000 Transfection Reagent (Invitrogen, life technologies) was used according the manufacturer's instructions. Activities of Firefly and Renilla luciferase were measured sequentially 48 h post-transfection using the Dual-Luciferase reporter assay system (Promega) as recommended by

the manufacturer, on a LUMAT LB 9507 luminometer (EGG-Berthold). Results are presented as relative light units of Firefly luciferase activity over relative light units of Renilla luciferase activity to compensate for variations in transfection efficiency and results were plotted as a percentage of the activity measured in the appropriate control.

### **Cell proliferation analysis**

NCI H295R cells were plated at a density of  $4 \times 10^4$  cells per well in 96-well microplates one day before transfection. Each condition was performed in triplicate using 0.3  $\mu\text{l}$  Lipofectamine RNAiMAX per well and 50 nM of mirVANA miRNA inhibitors (for hsa-miR-483-5p and hsa-miR-139-5p) or miRNA mimics (for hsa-miR-195 and hsa-miR-335) or mirVANA miRNA negative control, diluted in complete medium without antibiotics up to a final volume of 100  $\mu\text{l}$  per well. One day later, cells were serum-starved overnight and then stimulated with 2% serum. After 24h-, 48h-, or 72h- incubation, 10  $\mu\text{l}$  of The Cell Proliferation reagent WST-1 (Roche Applied Science) was added into each well then cells were incubated at 37°C for 2 additional hours. Absorbance measurement of the formazan product was performed with a microplate reader (Thermo Scientific Multiskan EX) at 450 nm wavelength.

### **Cell cycle and apoptosis analysis**

Cell cycle was analyzed using propidium iodide (Sigma-Aldrich) staining 24h after transfection of NCI H295R cells with 50 nM of microRNAs inhibitors, mimics, or miRNA negative control in 6-well plates. Cells were trypsinized, washed in PBS and centrifuged for 5 min at 1000 rpm then the pellet was placed on ice for 15 min. Cells were re-suspended in 400  $\mu\text{l}$  of staining solution containing 10  $\mu\text{l}$  of RNase 1mg.ml<sup>-1</sup>, 10  $\mu\text{l}$  of propidium iodide, and 380  $\mu\text{l}$  of 0.1% (w/v) Tri-sodium citrate dihydrate and 0.1% (v/v) Triton X-100 (Sigma-Aldrich), and incubated protected from light at 4°C overnight.

Flow cytometric measurement was performed on a FACSCalibur instrument (BD Biosciences) and analyses were performed for 20 000 events (cells) using CellQuest software. Apoptosis analysis was performed by staining cells in 100 µl of 1X Annexin V Binding Buffer containing 5 µl of FITC Annexin V and 5 µl of propidium iodide (FITC Annexin V Apoptosis Detection Kit I, BD Pharmingen) according to the manufacturer's instructions. Cells were then analyzed within 1 hour using a flow cytometer as described above.

### **Transwell migration and invasion assay**

In vitro cell migration assays were performed using translucent Transwell chambers (8 µm-pore size, BD Falcon Cell Culture Inserts). The lower face of the membrane was coated with rat tail Collagen I (BD Biosciences). NCI H295R cells were transfected in 12-well plates with 50 nM of miRNAs inhibitors, or miRNA negative control. 24h after transfection, the cells were trypsinized, resuspended in 0.5% serum containing medium and  $15 \times 10^3$  cells (300µl) were added to the upper chamber placed in 24-well plates. Complete medium containing 15% of Cosmic Calf Serum was added to the bottom chambers (750 µl). After 24 hours of migration, cells that had not migrated to the lower surface of the membrane were removed from the upper face of the membrane using cotton swabs. Cells that had migrated were fixed for 20 min using 4% Paraformaldehyde solution (Sigma) and stained for 30 min using a Crystal Violet solution (0.005% in 10% methanol). Images of five random fields ( $\times 10$ ) on each membrane were captured, and the number of migratory cells was counted; the mean values of three duplicate assays for each experimental condition were used for statistical analysis. Invasion assay was performed using inserts coated with 100µl of Matrigel Basement Membrane Matrix (BD Biosciences) diluted at 1:25 with DMEM/F-12.  $3 \times 10^4$  cells were seeded in 300µl of serum-free medium in the upper chambers whereas 15%-serum containing medium was added in the bottom chambers. Cells were fixed and stained 4-5 days later as described above.

### **Anchorage-independent colony formation assay in soft agar**

Soft agar plates were prepared in 12-well plates using a bottom layer containing 0.6 % (w/v) of Noble Agar (Gibco, Becton Dickinson) dissolved in complete growth medium for NCI H295R (DMEM/F-12, 5% Cosmic Calf Serum, 1% ITS, antibiotics). NCI H295R cells were transfected with miRNA inhibitors and trypsinized, then 15 000 cells were suspended in 0.3 % (w/v) of soft agar in complete growth medium, and plated as a top layer. 250 µl of growth medium were added once a week. Colony formation was monitored over a period of 14 days. For colony counting, growth medium was replaced by 500 µl of 0.0001 % crystal violet in 10 % ethanol. After 2 h, the staining solution was removed and each well was carefully rinsed several times with tap water until the soft agar became nearly transparent. The total number of colonies above 6 µm was then counted using Image J Software.

### **Statistical analyses**

Statistical analyses were performed with GraphPad Prism software version 4.03 (San Diego, California, USA) using one-way ANOVA or t tests where appropriate. Results are expressed as mean  $\pm$  SE or mean  $\pm$  SD. Statistical significance is indicated as \* for  $p \leq 0.05$ ; \*\* for  $p \leq 0.01$ ; \*\*\* for  $p \leq 0.001$ .

## RESULTS

### miRNAs expression profile in tumor tissues and identification of putative target genes

We focused on the four microRNAs that we have previously shown to be differentially expressed in ACCs compared with ACAs and that appeared as potential biomarkers for diagnosis and prognosis (28). This study was based on a microarray analysis followed by RT-qPCR validation, in two independent cohorts of 18 and 28 adrenocortical tumors. The level of miR-195 and miR-335 was found significantly decreased in both non-aggressive and aggressive ACC (naACCs and aACCs) as compared to ACA and normal adrenal cortex tissues, whereas miR-483-5p was markedly overexpressed in all ACCs. Interestingly, miR-139-5p expression level was reported to be up-regulated only in aACC tumors compared to naACC (28). To identify putative gene targets of the four differentially expressed miRNAs, we combined our data from an *in silico* analysis using different bioinformatics algorithms and data from gene expression analysis of adrenocortical tumors (34). Results are summarized in **Table 1**. We focused our further experiments on potential mRNA/miRNA interactions between miR-483-5p and miR-139-5p and two unidentified genes in the physiopathology of adrenocortical tumors: the N-myc Downstream Regulated Gene family member 2 (NDRG2) as a target of miR-483-5p and the family member 4 (NDRG4) as a target of miR-139-5p. These two genes were found down-regulated in the transcriptomic analysis of adrenocortical tumors (**Table 1**) whereas miR-483-5p and miR-139-5p expression levels were found up-regulated in the miRNome analysis (with mRNA or microRNA fold-change greater than 2-fold). **Figure 1** shows the miRNA/mRNA target physical interaction and secondary structures predicted by RNAHybrid software, combined with the results from miRWalk and TargetScan prediction softwares.

## **Inverse correlation between NDRG2/NDRG4 expression and miR-483-5p/miR-139-5p expression levels in tumor tissues**

To validate the data obtained with the microarray analyses, we measured the mRNA expression level of NDRG2 and NDRG4 by RT-qPCR in a cohort of 30 adrenocortical tissues including 18 carcinomas, 10 adenomas and 3 normal cortical tissues. We found a down-regulation of NDRG2 in aggressive ACCs compared with adenomas ( $p<0.001$ ) and with normal tissues ( $p<0.05$ ) whereas no significant difference was observed between non-aggressive ACCs and adenomas (**Figure 2B**). Importantly, NDRG4 mRNA was significantly down-regulated in aACC when compared with naACC ( $p<0.001$ ; **Figure 2B**). As shown in **Figure 2A**, low NDRG2 expression was associated with high miR-483-5p expression in aACC whereas a high NDRG2 expression was associated with a low miR-483-5p expression in ACA. Similarly, low NDRG4 expression was associated with high miR-139-5p expression in aACC, as compared with naACC (**Figure 2A**). Interestingly, expression levels of NDRG2 and NDRG4 were dramatically low in the human NCI H295R adrenocortical carcinoma cell line and were comparable to those measured in aACC tumors (**Figure 2B**). **Figure 2C** represents the correlation between the expression of microRNAs and putative target genes in each aACC or naACC. Significant inverse correlations between expression levels of NDRG2 and miR-483-5p, and between expression levels of NDRG4 and miR-139-5p were found in aACC but not in naACC tumors.

## **Effect of miR-483-5p and miR-139-5p inhibitors on NDRG expression in NCI H295R cells**

In light of the above results, we hypothesized that NDRG2 and NDRG4 might be target genes of miR-483-5p and miR-139-5p, respectively. We first confirmed by real-time PCR that miR-483-5p and miR-139-5p were overexpressed in NCI H295R: the normalized expressions of miR-483-5p and miR-139-5p were 90-fold and 18-fold higher, respectively, in NCI H295R cells compared with

normal adrenocortical tissues (**Figure 3A**). These results led us to examine whether inhibition of miR-483-5p and miR-139-5p could restore endogenous expression of NDRG2 and NDRG4 mRNAs, respectively, in NCI H295R cells (**Figure 3B**). To this end, miR-483-5p and miR-39-5p expression was down-regulated using specific inhibitors in NCI H295R cells. **Figure 3C** shows that the levels of miR-483-5p and miR-139-5p were efficiently decreased respectively by 3.3 and 8.6-fold within 24h as compared to Negative Control (NC) miRNA inhibitor and that inhibition was maintained at least up to 72h in NCI H295R cells. Quantitative PCR analysis revealed that miR-483-5p and miR-139-5p blockade led to re-expression of NDRG2 and NDRG4 mRNA 72h post-transfection (**Figure 3D**). These results suggest that miR-483-5p and miR-139-5p regulate NDRG2 and NDRG4 expression, respectively, at the post-transcriptional level. Analysis of NDRG2 and NDRG4 at the protein level in the same experimental conditions did not show any significant up-regulation of both proteins (not shown). Therefore, NCI H295R cells were transiently transfected with miR-483-5p or miR-139-5p mimics to assess the effect on endogenous NDRG protein expression. As shown in **Figure 3E**, miR-139-5p overexpression decreased NDRG4 whereas miR-483-5p decreased NDRG2 protein levels. Unexpectedly, miR-139-5p also decreased NDRG2 protein expression. These results suggest that NDRG2 mRNA levels may be modulated by both miRNAs.

### **MiR-483-5p and miR-139-5p functionally interact with the 3'-untranslated region of NDRG2 and NDRG4, respectively**

We then generated reporter constructs in which the wild-type 3'-untranslated region containing the predicted miRNA binding site was cloned downstream of the luciferase reporter gene (pMIR-Luc-3'UTR constructs). To investigate whether miR-483-5p and miR-139-5p target the 3'UTR of their putative mRNA targets, miR-483-5p or miR-139-5p inhibitors or negative control inhibitors were co-transfected with these reporter plasmids into NCI H295R cells. **Figure 4A** shows that miR-483-5p

inhibitor significantly increased luciferase activity of pMIR-Luc-NDRG2 construct to 1.6-fold of luciferase activity measured in NC inhibitor-treated cells. On the other hand, miR-139-5p inhibitor increased the luciferase activity of pMIR-Luc-NDRG4 construct to 1.8-fold of the one measured in NC inhibitor-treated cells. Conversely, co-transfection experiments were performed with miR-483-5p and miR-139-5p mimics or miRNA negative control in the presence of reporter plasmids in HEK293 cells. **Figure 4B** shows that miR-483-5p significantly decreased the luciferase activity of pMIR-Luc-NDRG2 construct to 0.6-fold of luciferase activity in NC miR-treated cells and that miR-139-5p decreased the luciferase activity of pMIR-Luc-NDRG4 construct to 0.7-fold, suggesting that both miRNA target their predicted 3'UTR binding site. To determine whether these effects were due to a direct interaction between NDRG2 3'UTR and miR-483-5p (at the 787 bp position) and between NDRG4 3'UTR and miR-139-5p (at the 1522 bp position), we generated reporter constructs in which the 3'UTR region containing the predicted miRNA binding site was mutated (**Figure 2B**). **Figure 4B** shows that miR-483-5p and miR-139-5p transfection in HEK293 cells did not affect the luciferase activity of pMIR-Luc-NDRG2 and pMIR-luc-NDRG4 mutated constructs, respectively, suggesting that indeed miR-483-5p and miR-139-5p interact physically with their target 3'UTR.

#### **MiR-483-5p and miR-139-5p did not affect NCI H295R cell proliferation or apoptosis.**

As our results indicated that miR-483-5p and miR-139-5p were overexpressed in ACC (oncogenic miRNAs) and that, conversely, miR-195 and miR-335 were under-expressed (tumor suppressor miRNAs), we next investigated the role of these miRNAs in adrenocortical cell tumorigenesis. NCI R295R cells were transfected with miR-483-5p and miR-139-5p inhibitors or with miR-195 and miR-335 mimics. WST1 proliferation assay and cell cycle analysis showed no statistically significant differences between NCI H295R cells transfected with miR-483-5p and miR-139-5p inhibitors or with miR-195 and miR-335 mimics and cells transfected with miRNA negative controls or mock-

transfected ones (**Figure 5**). Similarly, Annexin V-based cell apoptosis assays showed no significant differences between miR-483-5p or miR-139-5p inhibitor-transfected cells, miR-195 or miR-335 mimic-transfected cells and miRNA negative control-transfected cells (**Figure 6**). These results indicate that miR-483-5p and miR-139-5p are not involved in the regulation of NCI H295R proliferation or apoptosis in two-dimensional growth assays.

### **MiR-483-5p and miR-139-5p promote migration and invasion of NCI H295R cells**

To evaluate the potential role of miR-483-5p and miR-139-5p in ACC aggressiveness, we analyzed the migration and invasion capacities of NCI H295R using Boyden chamber-based assay (Transwell Assay as described in Material and Methods). **Figure 7A** shows that the migration of NCI H295R cells transfected with miR-483-5p or miR-139-5p inhibitors through the insert membrane was significantly reduced. Furthermore, miR-483-5p or miR-139-5p inhibitors also induced a marked inhibition of NCI H295R cell invasion through the Matrigel, when compared to negative control miR inhibitor. As both miRNAs target NDRG2 and NDRG4, we evaluated whether restoration of NDRG2 and NDRG4 could affect invasion capacities of NCI H295R cells. **Figure 7B** shows that the invasion of NCI H295R cells transfected with NDRG2 or NDRG4 constructs was reduced as compared to control cells, suggesting that NCI invasiveness is mediated, at least in part, through down-regulation of NDRG2 and NDRG4 by miR-483-5p and miR-139-5p.

### **MiR-483-5p and miR-139-5p are involved in anchorage-independent colony formation of NCI H295R cells**

Anchorage-independent growth (AIG) is a common characteristic of cancer cells. To determine the effect of miR-483-5p or miR-139-5p silencing on AIG in NCI H295R cells, miRNA inhibitor-transfected cells were analyzed for their ability to growth in soft agar (**Figure 8**). Although miR-

483-5p or miR-139-5p inhibitors had no significant effect on NCI H295R two-dimensional monolayer growth (**Figure 5**), the number and size of colonies were decreased when the cells were grown in soft agar, suggesting that both miRNAs are involved in loss of contact inhibition in adrenocortical cancer cells. These results point a role of miR-483-5p or miR-139-5p overexpression in the aggressive phenotype of NCI H295R cells.

## *DISCUSSION*

ACC is rare and highly aggressive malignancy, associated with a pejorative prognosis due to distant metastasis in most cases. Unfortunately, current treatment fails in many patients with advanced ACC, including the first line of combined chemotherapy currently used in metastatic tumors (37). The underlying molecular mechanisms resulting in carcinogenesis and aggressiveness remain obscure and stand for the major limitation in finding novel molecular-targeted strategies (5). So far, therapies targeting VEGF and its receptors, or multi-tyrosine kinase inhibitors failed to demonstrate clinical efficacy in ACC patients (4,9,11,12). Hopes were pinned on drugs targeting the insulin growth factor 2 (IGF-2), the most up-regulated gene in ACC, and its receptor IGF-1R, but a recent phase 3 trial showed disappointing results (38). Thus, networking and progress in the molecular characterization of ACC are ongoing thanks to the ENS@T network and The Cancer Genome Atlas (TCGA) international consortium. Researchers are developing molecular tools to better identify potential targets for personalized treatment, potential prognostic markers and predictive factors of new treatments efficacy, in order to further introduce them into the clinics (13,17). Several studies are ongoing to improve the classification of adrenocortical tumors based on pan-genomic analysis of genetic mutations, chromosomal aberrations, DNA methylation (the methylome) and gene expression (the transcriptome). The integration of microRNA expression (the miRNome) is an important part of this project.

In our study, we found that miR-483-5p and miR-139-5p target potential tumor suppressors, NDRG2 and NDRG4, respectively. Furthermore, miR-483-5p and miR-139-5p inhibition could effectively suppress the invasive potential of adrenocortical carcinoma cells in vitro. Thus, miR-483-5p and miR-139-5p might act as oncomiRNAs in ACC pathogenesis and represent potential therapeutic targets for this highly aggressive and therapy-refractory tumor.

Genetic aberration patterns have been defined previously and even if there are considered as early or late events occurring in ACC tumorigenesis, they are not sufficient to explain the entire malignant transformation process and chronology (13). Numerous studies have shown an overexpression of IGF2, a constitutive activation of Wnt/beta-catenin pathway, and an inactivation of the p53 suppressor gene in most ACCs. Abnormalities in the imprinted 11p15 region (involving maternal 11p15 Loss of Heterozygosity (LOH) with duplication of the active IGF-2 paternal allele) result in strong overexpression of IGF-2 and occur in more than 85% of ACCs. The markedly elevated IGF2 in sporadic ACC is likely to involve additional mechanisms of transcriptional regulation (14,15). Interestingly, miR-483-5p which was previously found to be overexpressed in ACC tumors compared with ACAs, resides within the second intron of IGF-2 and both were found significantly co-expressed in ACC tumors (24,26,28,29).

We focused our analysis on the most deregulated miRNA in ACC, according to our previous study: miR-195 and miR-335, which are underexpressed in ACC tumors compared with ACA, and miR-483-5p and miR-139-5p which are overexpressed (28). Using several prediction softwares, we identified several potential gene targets. Integrating the transcriptome and the miRNome data of ACCs allowed us to select gene targets inversely correlated with miRNA expression profile in ACCs. Surprisingly, when we restore the expression of miR-195 and miR-335 in NCI H295R cells, we did not observe any significant effect on the putative gene target expression (data not shown). Therefore, molecular mechanisms involved in the dysregulation of these miRNAs in ACC and their molecular targets remain to be explored further. We observed an increase in NDRG2 and NDRG4 expression mRNA levels by suppressing miR-483-3p or miR-139-5p expression in the human ACC cell line NCI-H295R. In addition, the functional interaction between miR-483-5p and NDRG2, and between miR-139-5p and NDRG4, was confirmed by luciferase reporter assays, indicating that each miRNA post-transcriptionally regulate NDRG2 and NDRG4 expression via a binding site in their 3'UTR

region. Interestingly, the mutation of the specific miRNA binding region of NDRG2- or NDRG4-3'UTR showed no effect on luciferase activity, indicating a compromised interaction between both microRNAs and their target gene. Unexpectedly and by contrast to the results obtained for NDRG2/4 mRNA levels, we did not find any significant increases in NDRG2/4 protein expression in NCI H295R cells transfected with miRNA inhibitors (data not shown). However, it is worth mentioning that the basal endogenous miR-483-5p and miR-139-5p levels are very high in NCI H295R cells compared with normal adrenal cortex (100- and 20-fold increase in NCI H295R cells, respectively). Consequently, substantial amounts of endogenous miRNA remain available within the cells despite miRNA inhibition. We therefore decided to assess endogenous NDRG protein expression using miRNA mimics. We could observe a significant decrease in NDRG4 and NDRG2 protein expression following miR-139-5p and miR-483-5p overexpression. Unexpectedly, miR-139-5p also decreased NDRG2 protein levels. Using prediction softwares, we found that miR-139-5p was predicted to bind the 3'UTR region of NDRG2, although with a lower score than miR-483-5p. These observations confirm that multiple miRNAs can target the same gene and suggest that the combination of miR-139-5p and miR-483-5p activities might determine the expression of NDRG2 in NCI H295R cells.

We demonstrated that NDRG2 expression in human adrenal tumors was significantly lower in both aggressive and non-aggressive ACC compared with ACA. Furthermore, low NDRG4 levels could discriminate between aggressive ACC and non-aggressive ACC. These results need to be confirmed in larger cohorts of patients. We showed that low NDRG2 and NDRG4 expression levels in ACC were inversely correlated with high expression levels of miR-483-5p and miR-139-5p, respectively. NDRG2 and NDRG4 belong to the N-myc downstream regulated gene family of proteins consisting of 4 members, NDRG1 to NDRG4, which share 53-65% amino acid identity. **Figure 9** represents a literature overview on NDRG family functions. The NDRG genes are located on different chromosomes, and the distinct subcellular (nuclear versus cytoplasm) and tissue distributions of the

different members suggest that each of them may have different function. Although there is evidence that they play fundamental roles in physiological processes such as cell proliferation, differentiation, development, and stress responses, the exact function of these proteins needs to be explored further (39–41). The mRNA and protein level of NDRG2 has been shown to be significantly down-regulated in a variety of human malignant tumors such as breast cancer, colorectal adenocarcinoma, glioblastoma, hepatocellular carcinoma, thyroid cancer, pancreatic cancer and gastric cancer (42–46). Thus, several studies support a tumor suppressor role for NDRG2 in cancer. The downregulation of NDRG2 in malignant tumors was reported to be closely correlated with poor overall survival, metastasis, and highly invasive phenotype (47–50). Therefore, NDRG2 was considered as a potentially valuable prognostic marker in patients. There are less data available concerning NDRG4 expression in cancer, but it seems to have tumor-suppressive or oncogenic functions depending on the tissue type (51–53). The regulation of NDRG protein expression involves complex and various mechanisms including not only transcription repressors such as N-myc and C-myc proto-oncogenes, but also promoter hyper-methylation. Interestingly, NDRG2 promoter CpG island methylation and downregulation have been observed in many cancers including ACC (54–56). Our data highlight an additional mechanism for the regulation of NDRG2 and NDRG4 expression in ACCs, through miR-483-5p- and miR-139-5p-mediated post-transcriptional control. Similar mechanisms have been reported in recent studies showing that miR-122 in hepatocellular carcinoma, or miR-650 in colorectal carcinoma, repress the expression of NDRG3 and NDRG2, respectively (57,58).

Our data show that silencing miR-483-5p or miR-139-5p and overexpressing miR-195 or miR-335 in NCI-H295R cells did not affect cell proliferation or apoptosis. By contrast, a previous study reported that inhibition of miR-483-5p or overexpression of miR-195 in NCI-H295 cells resulted in a significant reduction of cell proliferation (23). Although we do not have explanation for these discrepancies, one should mention that in Ozata's study, the NCI H295R cells were electroporated

with 20-times higher concentrations (1  $\mu$ M) of miRNA inhibitors or miRNA mimics, which could lead to non-specific effects on gene expression.

We observed that miR-483-5p inhibitor significantly decreased NCI H295R cell migration and invasion. These results are in agreement with those reported very recently by Song and co-workers, who demonstrated that miR-483-5p is a critical prometastatic miRNA in lung adenocarcinoma (59). We found that miR-139-5p inhibition also disrupts NCI H295R cell invasion and migration. This is contrast to the data reported by Krishnan et al demonstrating that miR-139-5p overexpression plays an anti-invasive role in breast cancer (60). However, one should keep in mind that miRNAs can play tumor suppressive or oncogenic functions depending on the cell context. As we have shown in our study that NDRG2 and NDRG4 mRNA expression is dramatically decreased in ACC as compared to normal adrenal cortex and as both transcripts are targets of miR-483-5p and miR-139-5p, respectively, we re-expressed NDRG2 and NDRG4 in NCI H295R cells and could show that invasion capacities of NCI H295R cells were impaired.

Anchorage-independent growth in soft agar, which is considered as the most stringent assay for detecting malignant cell transformation, revealed that miR-483-5p or miR-139-5p inhibition induced a marked decrease in the number and the size of the colonies compared with negative controls. These results suggest that both microRNAs are involved in phenotypic changes of NCI H295R cells such as loss of contact inhibition and cell ability to grow in a viscous fluid or gel. This process is assumed to be closely related to the *in vivo* carcinogenesis. *In vivo* strategies based on exogenous miRNA delivery (mimic or inhibitor) in cancer models remain the key challenge for the establishment of this novel class of RNA therapeutics (32,61). Therefore, our results are promising in the prospect of testing the effect of miR-483-5p and miR-139-5p inhibitors as therapeutic tools in an ACC model in mice. There are several major difficulties to overcome before considering modulating endogenous microRNA expression in patients, such as technical issues (administration mode etc.) and also

potential off-target actions and side effects due to the tissue-specific nature of microRNA action. The pathways affected by microRNA dysregulation in ACC tumors could also serve as indirect therapeutic targets (22). In this study, we identified for the first time two target genes among the NDRG family, NDRG2 for miR-483-5P and NDRG4 for miR-139-5p, that play a tumor suppressor role in ACC tumors and participate to the invasive phenotype of NCI H295R. We hypothesize that the downstream pathways altered by both oncomiRs and their targets involves the Epithelial-Mesenchymal Transition (EMT) which is required for metastasis, and largely influenced by the WNT/Beta-catenin signaling (62,63). Recent studies have shown a correlation between loss of NDRG2 and invasive characteristics of different cancer cell models and suggested an important role for EMT as a molecular mechanism involved in these processes (64–66). Further experiments have to be performed to explore this hypothesis.

Moreover, the recent discovery of the overexpression of miR-483-5p and miR-139-5p in the serum of patients affected by ACC opens the way for the study of their possible role as intercellular communication molecules (28). MiR-139-5p and particularly miR-483-5p were found highly abundant in the culture medium of NCI H295R cells (data not shown), in line with what has been observed in the serum of ACC patients. Tumor-derived circulating miRNAs may be transferred to recipient cells such as endothelial cells or other cells of the tumor micro-environment and may remodel their gene expression. This could promote angiogenesis, and lead to vascular endothelial barrier destruction and metastasis of tumor cells. Our preliminary results open promising perspectives in this field.

## RÉFÉRENCES

1. Kebebew E, Reiff E, Duh Q-Y, Clark OH, McMillan A. Extent of disease at presentation and outcome for adrenocortical carcinoma: have we made progress? *World J Surg.* mai 2006;30(5):872-8.
2. Fassnacht M, Johanssen S, Quinkler M, Bucsky P, Willenberg HS, Beuschlein F, et al. Limited prognostic value of the 2004 International Union Against Cancer staging classification for adrenocortical carcinoma: proposal for a Revised TNM Classification. *Cancer.* 15 janv 2009;115(2):243-50.
3. Lughezzani G, Sun M, Perrotte P, Jeldres C, Alasker A, Isbarn H, et al. The European Network for the Study of Adrenal Tumors staging system is prognostically superior to the international union against cancer-staging system: a North American validation. *Eur J Cancer Oxf Engl* 1990. mars 2010;46(4):713-9.
4. Else T, Kim AC, Sabolch A, Raymond VM, Kandathil A, Caoili EM, et al. Adrenocortical carcinoma. *Endocr Rev.* avr 2014;35(2):282-326.
5. Fassnacht M, Kroiss M, Allolio B. Update in adrenocortical carcinoma. *J Clin Endocrinol Metab.* déc 2013;98(12):4551-64.
6. Libé R. Adrenocortical carcinoma (ACC): diagnosis, prognosis, and treatment. *Front Cell Dev Biol.* 2015;3:45.
7. Mihai R. Diagnosis, treatment and outcome of adrenocortical cancer. *Br J Surg.* mars 2015;102(4):291-306.
8. Libé R, Borget I, Ronchi CL, Zaggia B, Kroiss M, Kerkhofs T, et al. Prognostic factors in stage III-IV adrenocortical carcinomas (ACC): an European Network for the Study of Adrenal Tumor (ENSAT) study. *Ann Oncol Off J Eur Soc Med Oncol ESMO.* oct 2015;26(10):2119-25.
9. Kirschner LS. The next generation of therapies for adrenocortical cancers. *Trends Endocrinol Metab TEM.* juill 2012;23(7):343-50.
10. Lehmann T, Wrzesinski T. The molecular basis of adrenocortical cancer. *Cancer Genet.* avr 2012;205(4):131-7.
11. Ragazzon B, Assié G, Bertherat J. Transcriptome analysis of adrenocortical cancers: from molecular classification to the identification of new treatments. *Endocr Relat Cancer.* avr 2011;18(2):R15-27.
12. Stigliano A, Cerquetti L, Sampaoli C, Bucci B, Toscano V. Current and emerging therapeutic

- options in adrenocortical cancer treatment. *J Oncol.* 2012;2012:408131.
13. Assié G, Jouinot A, Bertherat J. The « omics » of adrenocortical tumours for personalized medicine. *Nat Rev Endocrinol.* avr 2014;10(4):215-28.
14. Gicquel C, Bertagna X, Gaston V, Coste J, Louvel A, Baudin E, et al. Molecular markers and long-term recurrences in a large cohort of patients with sporadic adrenocortical tumors. *Cancer Res.* 15 sept 2001;61(18):6762-7.
15. Giordano TJ, Kuick R, Else T, Gauger PG, Vinco M, Bauersfeld J, et al. Molecular classification and prognostication of adrenocortical tumors by transcriptome profiling. *Clin Cancer Res Off J Am Assoc Cancer Res.* 15 janv 2009;15(2):668-76.
16. Assié G, Letouzé E, Fassnacht M, Jouinot A, Luscap W, Barreau O, et al. Integrated genomic characterization of adrenocortical carcinoma. *Nat Genet.* juin 2014;46(6):607-12.
17. De Martino MC, Al Ghuzlan A, Aubert S, Assié G, Scoazec J-Y, Leboulleux S, et al. Molecular screening for a personalized treatment approach in advanced adrenocortical cancer. *J Clin Endocrinol Metab.* oct 2013;98(10):4080-8.
18. Drougat L, Omeiri H, Lefèvre L, Ragazzon B. Novel Insights into the Genetics and Pathophysiology of Adrenocortical Tumors. *Front Endocrinol.* 2015;6:96.
19. Ragazzon B, Libé R, Gaujoux S, Assié G, Fratticci A, Launay P, et al. Transcriptome analysis reveals that p53 and {beta}-catenin alterations occur in a group of aggressive adrenocortical cancers. *Cancer Res.* 1 nov 2010;70(21):8276-81.
20. Bezerra JE, Latronico AC. MicroRNA era: the importance for diagnosis and prognosis of adrenocortical tumors. *BioMed Res Int.* 2014;2014:381917.
21. Duregon E, Rapa I, Votta A, Giorcelli J, Daffara F, Terzolo M, et al. MicroRNA expression patterns in adrenocortical carcinoma variants and clinical pathologic correlations. *Hum Pathol.* août 2014;45(8):1555-62.
22. Igaz P, Igaz I, Nagy Z, Nyírő G, Szabó PM, Falus A, et al. MicroRNAs in adrenal tumors: relevance for pathogenesis, diagnosis, and therapy. *Cell Mol Life Sci CMLS.* févr 2015;72(3):417-28.
23. Özata DM, Caramuta S, Velázquez-Fernández D, Akçakaya P, Xie H, Höög A, et al. The role of microRNA deregulation in the pathogenesis of adrenocortical carcinoma. *Endocr Relat Cancer.* oct 2011;18(6):643-55.
24. Patterson EE, Holloway AK, Weng J, Fojo T, Kebebew E. MicroRNA profiling of adrenocortical tumors reveals miR-483 as a marker of malignancy. *Cancer.* 15 avr 2011;117(8):1630-9.
25. Schmitz KJ, Helwig J, Bertram S, Sheu SY, Suttorp AC, Seggewiss J, et al. Differential

expression of microRNA-675, microRNA-139-3p and microRNA-335 in benign and malignant adrenocortical tumours. *J Clin Pathol.* juin 2011;64(6):529-35.

26. Soon PSH, Tacon LJ, Gill AJ, Bambach CP, Sywak MS, Campbell PR, et al. miR-195 and miR-483-5p Identified as Predictors of Poor Prognosis in Adrenocortical Cancer. *Clin Cancer Res Off J Am Assoc Cancer Res.* 15 déc 2009;15(24):7684-92.
27. Wang C, Sun Y, Wu H, Zhao D, Chen J. Distinguishing adrenal cortical carcinomas and adenomas: a study of clinicopathological features and biomarkers. *Histopathology.* mars 2014;64(4):567-76.
28. Chabre O, Libé R, Assie G, Barreau O, Bertherat J, Bertagna X, et al. Serum miR-483-5p and miR-195 are predictive of recurrence risk in adrenocortical cancer patients. *Endocr Relat Cancer.* août 2013;20(4):579-94.
29. Patel D, Boufraqech M, Jain M, Zhang L, He M, Gesuwan K, et al. MiR-34a and miR-483-5p are candidate serum biomarkers for adrenocortical tumors. *Surgery.* déc 2013;154(6):1224-8; discussion 1229.
30. Szabó DR, Luconi M, Szabó PM, Tóth M, Szücs N, Horányi J, et al. Analysis of circulating microRNAs in adrenocortical tumors. *Lab Investig J Tech Methods Pathol.* mars 2014;94(3):331-9.
31. Singh P, Soon PSH, Feige J-J, Chabre O, Zhao JT, Cherradi N, et al. Dysregulation of microRNAs in adrenocortical tumors. *Mol Cell Endocrinol.* 31 mars 2012;351(1):118-28.
32. Schoof CRG, Botelho EL da S, Izzotti A, Vasques LDR. MicroRNAs in cancer treatment and prognosis. *Am J Cancer Res.* 2012;2(4):414-33.
33. Ritchie W, Rasko JEJ. Refining microRNA target predictions: Sorting the wheat from the chaff. *Biochem Biophys Res Commun.* 21 mars 2014;445(4):780-4.
34. de Reyniès A, Assié G, Rickman DS, Tissier F, Groussin L, René-Corail F, et al. Gene expression profiling reveals a new classification of adrenocortical tumors and identifies molecular predictors of malignancy and survival. *J Clin Oncol Off J Am Soc Clin Oncol.* 1 mars 2009;27(7):1108-15.
35. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 15 août 1970;227(5259):680-5.
36. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A.* sept 1979;76(9):4350-4.
37. Fassnacht M, Terzolo M, Allolio B, Baudin E, Haak H, Berruti A, et al. Combination chemotherapy in advanced adrenocortical carcinoma. *N Engl J Med.* 7 juin 2012;366(23):2189-97.

38. Fassnacht M, Berruti A, Baudin E, Demeure MJ, Gilbert J, Haak H, et al. Linsitinib (OSI-906) versus placebo for patients with locally advanced or metastatic adrenocortical carcinoma: a double-blind, randomised, phase 3 study. *Lancet Oncol.* avr 2015;16(4):426-35.
39. Yao L, Zhang J, Liu X. NDRG2: a Myc-repressed gene involved in cancer and cell stress. *Acta Biochim Biophys Sin.* juill 2008;40(7):625-35.
40. Melotte V, Qu X, Ongenaert M, van Criekinge W, de Bruïne AP, Baldwin HS, et al. The N-myc downstream regulated gene (NDRG) family: diverse functions, multiple applications. *FASEB J Off Publ Fed Am Soc Exp Biol.* nov 2010;24(11):4153-66.
41. Hwang J, Kim Y, Kang HB, Jaroszewski L, Deacon AM, Lee H, et al. Crystal structure of the human N-Myc downstream-regulated gene 2 protein provides insight into its role as a tumor suppressor. *J Biol Chem.* 8 avr 2011;286(14):12450-60.
42. Hu X-L, Liu X-P, Lin S-X, Deng Y-C, Liu N, Li X, et al. NDRG2 expression and mutation in human liver and pancreatic cancers. *World J Gastroenterol WJG.* 1 déc 2004;10(23):3518-21.
43. Tepel M, Roerig P, Wolter M, Gutmann DH, Perry A, Reifenberger G, et al. Frequent promoter hypermethylation and transcriptional downregulation of the NDRG2 gene at 14q11.2 in primary glioblastoma. *Int J Cancer J Int Cancer.* 1 nov 2008;123(9):2080-6.
44. Zhao H, Zhang J, Lu J, He X, Chen C, Li X, et al. Reduced expression of N-Myc downstream-regulated gene 2 in human thyroid cancer. *BMC Cancer.* 2008;8:303.
45. Kim Y-J, Yoon SY, Kim J-T, Song EY, Lee HG, Son HJ, et al. NDRG2 expression decreases with tumor stages and regulates TCF/beta-catenin signaling in human colon carcinoma. *Carcinogenesis.* avr 2009;30(4):598-605.
46. Lorentzen A, Lewinsky RH, Bornholdt J, Vogel LK, Mitchelmore C. Expression profile of the N-myc Downstream Regulated Gene 2 (NDRG2) in human cancers with focus on breast cancer. *BMC Cancer.* 2011;11:14.
47. Cao W, Yu G, Lu Q, Zhang J. Low expression of N-myc downstream-regulated gene 2 in oesophageal squamous cell carcinoma correlates with a poor prognosis. *BMC Cancer.* 2013;13:305.
48. Li S-J, Wang W-Y, Li B, Chen B, Zhang B, Wang X, et al. Expression of NDRG2 in human lung cancer and its correlation with prognosis. *Med Oncol Northwood Lond Engl.* mars 2013;30(1):421.
49. Oh S, Kim D, Kim D-H, Chang HH, Sohn K-C, Kim KH, et al. NDRG2 correlated with favorable recurrence-free survival inhibits metastasis of mouse breast cancer cells via attenuation of active TGF- $\beta$  production. *Carcinogenesis.* oct 2012;33(10):1882-8.
50. Yamamura A, Miura K, Karasawa H, Morishita K, Abe K, Mizuguchi Y, et al. Suppressed expression of NDRG2 correlates with poor prognosis in pancreatic cancer. *Biochem Biophys Res*

Commun. 8 nov 2013;441(1):102-7.

51. Chu D, Zhang Z, Zhou Y, Li Y, Zhu S, Zhang J, et al. NDRG4, a novel candidate tumor suppressor, is a predictor of overall survival of colorectal cancer patients. *Oncotarget*. 10 avr 2015;6(10):7584-96.
52. Kotipatruni RP, Ren X, Thotala D, Jaboin JJ. NDRG4 is a novel oncogenic protein and p53 associated regulator of apoptosis in malignant meningioma cells. *Oncotarget*. 10 juill 2015;6(19):17594-604.
53. Yang X, An L, Li X. NDRG3 and NDRG4, two novel tumor-related genes. *Biomed Pharmacother Bioméd Pharmacothérapie*. sept 2013;67(7):681-4.
54. Liu N, Wang L, Liu X, Yang Q, Zhang J, Zhang W, et al. Promoter methylation, mutation, and genomic deletion are involved in the decreased NDRG2 expression levels in several cancer cell lines. *Biochem Biophys Res Commun*. 22 juin 2007;358(1):164-9.
55. Barreau O, Assié G, Wilmot-Roussel H, Ragazzon B, Baudry C, Perlemoine K, et al. Identification of a CpG island methylator phenotype in adrenocortical carcinomas. *J Clin Endocrinol Metab*. janv 2013;98(1):E174-84.
56. Chang X, Li Z, Ma J, Deng P, Zhang S, Zhi Y, et al. DNA methylation of NDRG2 in gastric cancer and its clinical significance. *Dig Dis Sci*. mars 2013;58(3):715-23.
57. Fan C-G, Wang C-M, Tian C, Wang Y, Li L, Sun W-S, et al. miR-122 inhibits viral replication and cell proliferation in hepatitis B virus-related hepatocellular carcinoma and targets NDRG3. *Oncol Rep*. nov 2011;26(5):1281-6.
58. Feng L, Xie Y, Zhang H, Wu Y. Down-regulation of NDRG2 gene expression in human colorectal cancer involves promoter methylation and microRNA-650. *Biochem Biophys Res Commun*. 25 mars 2011;406(4):534-8.
59. Song Q, Xu Y, Yang C, Chen Z, Jia C, Chen J, et al. miR-483-5p Promotes Invasion and Metastasis of Lung Adenocarcinoma by Targeting RhoGDI1 and ALCAM. *Cancer Res*. 1 juin 2014;74(11):3031-42.
60. Krishnan K, Steptoe AL, Martin HC, Pattabiraman DR, Nones K, Waddell N, et al. miR-139-5p is a regulator of metastatic pathways in breast cancer. *RNA N Y N*. déc 2013;19(12):1767-80.
61. Pereira DM, Rodrigues PM, Borralho PM, Rodrigues CMP. Delivering the promise of miRNA cancer therapeutics. *Drug Discov Today*. mars 2013;18(5-6):282-9.
62. Ghahhari NM, Babashah S. Interplay between microRNAs and WNT/β-catenin signalling pathway regulates epithelial-mesenchymal transition in cancer. *Eur J Cancer Oxf Engl* 1990. août

2015;51(12):1638-49.

63. Kim M-J, Lim J, Yang Y, Lee M-S, Lim J-S. N-myc downstream-regulated gene 2 (NDRG2) suppresses the epithelial-mesenchymal transition (EMT) in breast cancer cells via STAT3/Snail signaling. *Cancer Lett.* 1 nov 2014;354(1):33-42.
64. Golestan A, Mojtabaei Z, Ghahamfarsa G, Hamidinia M, Takhshid MA. The Effects of NDRG2 Overexpression on Cell Proliferation and Invasiveness of SW48 Colorectal Cancer Cell Line. *Iran J Med Sci.* sept 2015;40(5):430-9.
65. Lee DG, Lee S-H, Kim J-S, Park J, Cho Y-L, Kim KS, et al. Loss of NDRG2 promotes epithelial-mesenchymal transition of gallbladder carcinoma cells through MMP-19-mediated Slug expression. *J Hepatol.* 17 août 2015;
66. Li R, Yu C, Jiang F, Gao L, Li J, Wang Y, et al. Overexpression of N-Myc downstream-regulated gene 2 (NDRG2) regulates the proliferation and invasion of bladder cancer cells in vitro and in vivo. *PloS One.* 2013;8(10):e76689.

## TABLE AND FIGURES

**Table 1. Putative miRNA/mRNA target interaction in ACC.**

mRNA targets of downregulated miRNAs (miR-195 and miR-335) were upregulated, whereas mRNA targets of upregulated miRNAs (miR-139-5p and miR-483-5p) were downregulated in aACC as compared to naACC. Of note, miR-483-5p was found markedly overexpressed in all carcinomas (naACC+aACC) *versus* adenomas using quantitative PCR (up to 100-fold, Chabre et al, 2013). mRNA and miRNA transcripts whose expression was changed by at least 2-fold were selected.

\**miRNA fold-changes according to our previous published miRNA expression profiling of adrenocortical tumors (Chabre et al. 2013).*

\*\**mRNA fold-changes according to previously published gene expression profiling of adrenocortical tumors (de Reynies et al, 2009).*

**p value:** probability distribution of random matches of a subsequence (miRNA 5'-end sequence) in the given sequence (mRNA 3'-end sequence) according to miRWALK prediction software. A  $p < 0.05$  implies a significant hit.

**Minimum Free Energy (MFE)** was provided by RNAHybrid algorithm (based on thermodynamic model), for prediction of miRNA-mRNA target interaction and secondary structure from the base sequence.

**Figure 1. Schematic representations of miR-483-5p and miR-139-5p interaction with NDRG2 and NDRG4 respectively, according to prediction softwares.**

**A,** miRNAs (in green) and mRNA target gene (in red) interaction and secondary structure using combined results from TargetScan, miRWALK and RNAHybrid prediction softwares.

**B**, NDRG2 or NDRG4 3'UTR pairing with miR-483-5p or miR-139-5p seed sequences, respectively. Nucleotides in red represent the mutations introduced in functional studies.

*5'-UTR: 5'-untranslated region; CDS: coding sequence*

**Figure 2. Inverse correlation between microRNAs expression and putative target gene expression in a cohort of human adrenocortical tissues.**

**A**, miRNA expression levels of miR-483-5p and miR-139-5p was measured by RT-qPCR in a cohort of human adrenocortical tissues from COMETE Network (10 adrenocortical adenomas (ACA), 9 non-aggressive adrenocortical carcinomas (naACA), 9 aggressive adrenocortical carcinomas (aACA), and 3 normal adrenocortical tissues (NA)). MiR-483-5p was up-regulated in ACC tumors compared with ACA, whereas miR-139-5p was up-regulated in aACC tumors compared with naACC.

**B**, NDRG2 and NDRG4 expression level was measured in the same cohort of patients by RT-qPCR. NDRG2 expression level was lower in aACC tissues compared with ACA and NA, whereas NDRG4 was under-expressed in aACC compared with naACC tissues.

**C**, Pearson correlation between expression levels of NDRG2 and miR-483-5p, and between expression levels of NDRG4 and miR-139-5p in aACC.

*Statistical analysis was performed using Kruskal-Wallis test, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.*

**Figure 3. Effect of miR-483-5p and miR-139-5p inhibitors on NDRG expression in NCI H295R cells.**

**A**, RT-qPCR analysis of endogenous microRNA expression level in NCI H295R cell line, normalized to RNU48 RNA and represented as a ratio of NCI H295R miRNA levels/normal adrenal miRNA

levels. MiR-139-5p and miR-483-5p are overexpressed by about 100- and 20-fold, respectively, as compared to normal adrenocortical cells.

**B**, Schematic representation of miRNA inhibitor mechanism of action.

**C and D**, NCI H295R cells were transiently transfected for 24 h, 48 h or 72 h with miR-483-5p inhibitor, miR-139-5p inhibitor, or Negative Control miR inhibitor. **C**, MiRNA repression was checked by RT-qPCR. **D**, Effect of miR-inhibitors on endogenous NDRG2 and NDRG4 mRNA expression at 72h (n=3).

**E**, Western-Blot analysis of NDRG2 and NDRG4 protein expression in NCI H295R transfected with 100 nM and 50 nM of miR-483-5p or miR-139-5p mimics, respectively. NDRG2 or NDRG4 expressions were normalized to Actin. Shown is a representative experiment of 2 independent ones.

**Figure 4. MiR-483-5p and miR-139-5p functionally interact with NDRG2 and NDRG4 3'UTR.**

**A**, MiR-483-5p and miR-139-5p inhibition increases NDRG2-3'UTR or NDRG4-3'UTR-driven luciferase activity in NCI H295R cells.

**B**, MiR-483-5p and miR-139-5p overexpression decreases NDRG2-3'UTR-Wild Type or NDRG4-3'UTR-Wild Type-driven luciferase activity in HEK293 cells. When mutations were introduced in the NDRG-3'UTR binding site, microRNAs overexpression in HEK293 cells had no effect on luciferase activity of pMIR-Luc-3'UTR-Mutant construct.

*Represented are Firefly/Renilla luciferase activity ratios. Statistical analysis was performed using ANOVA, \*\* p<0.01. Shown are the mean ± S.E.M of 4 independent experiment performed in duplicates for wild type Luc-3'UTR. Luciferase activity of Luc-3'UTR Mut constructs represents Mean ± SD from 2 experiments performed in duplicates.*

**Figure 5. Effect of miR-483-5p and miR-139-5p inhibition, or miR-195 and miR-335 overexpression, on cell cycle and cell proliferation in NCI-H295R cell line.**

MiRNA mimics (miR-195 and miR-335), miRNA inhibitors (miR-483-5p and miR-139-5p) or miR-negative control were transiently transfected in NCI-H295R cells.

**A**, Transfected and mock-transfected cells were stained with propidium iodide and analyzed by flow cytometry. The histogram shows the percentage of cells in the different cell cycle phases determined in each condition of transfection. No significant differences in cell cycle phases were observed between each condition (n=2).

**B**, Proliferation of NCI-H295 cells was measured by WST1 assays. 24 h, 48h and 72 h after transfection, WST1 was added in culture wells and formazan products were detected by colorimetric method reflecting cell viability. No statistical differences in cell proliferation rate were observed between each condition (n=4).

**Figure 6. Effect of miR-483-5p and miR-139-5p inhibition, or miR-195 and miR-335 overexpression, on NCI-H295R cell apoptosis.**

Cell apoptosis was assessed by Annexin V-FITC assay. MiRNA mimics (miR-195 and miR-335), miRNA inhibitors (miR-483-5p and miR-139-5p) or miR-negative control were transiently transfected in NCI-H295R cells. Transfected and mock-transfected cells were stained with Annexin V-FITC and propidium iodide (PI), and were analyzed by flow cytometry. The lower square on the right represent cells stained by Annexin V but not by PI (Annexin V+/PI-), reflecting cells in early stage of apoptosis. The upper square on the right reflects cells in late apoptosis and those undergoing necrosis or damaged cells (Annexin V+/PI+).

*No significant changes in the percentage of cells undergoing early apoptosis were observed between each condition (the rate of cells labeled Annexin V+/PI+ were the same in each culture condition). One representative of 2 independent experiments is shown (n=2).*

**Figure 7. Effect of miR-483-5p and miR-139-5p inhibition, or NDRG target gene overexpression on migration and invasion capacities of NCI-H295R cells.**

*Transwell assays were performed as described in Material and Methods to assess cell migration and invasion.*

**A**, NCI H295R cells were transiently transfected with negative control miR, miR-483-5p or miR-139-5p inhibitors, then analyzed in migration and invasion assays. The right panels are quantification of cell number in five random fields per well (mean ± SEM) and are representative of four independent experiments. **B**, Mock-transfected, NDRG2- or NDRG4-transfected NCI H295R cells (500 ng of plasmid) were analyzed in invasion assay. Western Blots on the right represent a dose-response for NDRG plasmids in NCI-H295R cells.

**Figure 8. Effect of miR-483-5p and miR-139-5p inhibition on anchorage-independent growth of NCI-H295R cells.**

Soft agar colony formation assay was performed as described in Materiel and Methods.

**A**, The colony size was greater in miR-Negative Control-transfected cells as compared to miR-inhibitors-transfected cells which also display lower colony-forming ability.

**B**, Quantification of colony number in 3 independent experiments.

\*\* p<0.01, \*\*\* p<0.001.

**Figure 9. The multiple functions of the N-Myc Downstream-Regulated gene (NDRG) family.**

The NDRG family is highly conserved between species. As they are repressed by the Myc oncogene, they have been argued to have tumor-suppressive and anti-metastatic functions. The NDRGs may play important roles in the development of cancer and other diseases. The molecular mechanisms underlying their diverse actions await further investigations.

From *Melotte et al. FASEB J. 2010* and *Hwang et al. J. Biol. Chem. 2011*

**Table 1. Putative miRNA/mRNA target interaction in ACC.**

| miRNA      | miRNA fold-change*<br>aACC vs<br>naACC (log2<br>ratio) | Putative<br>target<br>mRNAs<br>(negatively<br>correlated) | mRNA target<br>fold-change**<br>aACC vs<br>naACC<br>(log2 ratio) | p value | Minimum<br>free energy<br>(kcal/mole) | Description                                                |
|------------|--------------------------------------------------------|-----------------------------------------------------------|------------------------------------------------------------------|---------|---------------------------------------|------------------------------------------------------------|
| miR-195    | -1.2                                                   | WNT4                                                      | 2.4                                                              | 0.0410  | -25.1                                 | Wingless-type MMTV integration site family member 4        |
|            |                                                        | PRKD3                                                     | 2.0                                                              | 0.0401  | -26.0                                 | Protein kinase D3                                          |
|            |                                                        | CCND1                                                     | 1.2                                                              | 0.0478  | -24.8                                 | Cyclin D1                                                  |
| miR-335    | -2.3                                                   | COL6A3                                                    | 1.9                                                              | 0.0465  | -17.3                                 | Collagen type VI, alpha 3                                  |
|            |                                                        | TOP2A                                                     | 1.6                                                              | 0.0148  | -17.9                                 | Topoisomerase (DNA) II alpha                               |
| miR-139-5p | 6.0                                                    | NDRG4                                                     | -3.5                                                             | 0.0310  | -25.0                                 | Tumor suppressive N-Myc downstream-regulated gene member 4 |
| miR-483-5p | 1.6                                                    | NDRG2                                                     | -1.0                                                             | 0.0008  | -25.3                                 | Tumor suppressive N-Myc downstream-regulated gene member 2 |

\*miRNA fold-changes according to our previous published miRNA expression profiling of adrenocortical tumors (Chabre et al. 2013).

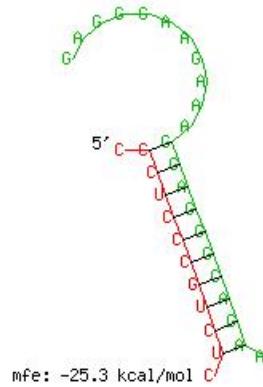
\*\*mRNA fold-changes according to previously published gene expression profiling of adrenocortical tumors (de Reynies et al, 2009).

**p value:** probability distribution of random matches of a subsequence (miRNA 5'-end sequence) in the given sequence (mRNA 3'-end sequence) according to miRWalk prediction software. A p<0.05 implies a significant hit.

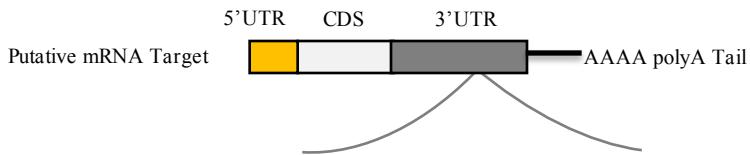
**Minimum Free Energy (MFE)** was provided by RNAHybrid algorithm (based on thermodynamic model), for prediction of miRNA-mRNA target interaction and secondary structure from the base sequence.

mRNA targets of downregulated miRNAs (miR-195 and miR-335) were upregulated, whereas mRNA targets of upregulated miRNAs (miR-139-5p and miR-483-5p) were downregulated in aACC as compared to naACC. Of note, miR-483-5p was found markedly overexpressed in all carcinomas (naACC+aACC) versus adenomas using quantitative PCR (up to 100-fold, Chabre et al, 2013).

mRNA and miRNA transcripts whose expression was changed by at least 2-fold were selected.

**A**

### miR-483-5p and NDRG2 3'UTR interaction

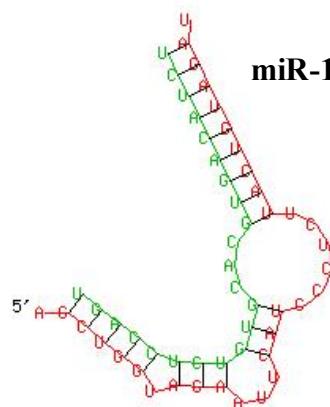
**B**

Seed sequence position 772-782 in the  
**Wild type NDRG2-3'UTR**

**hsa-miR-483-5p**

**Mutant NDRG2-3'UTR**

5'...UGUGGAGGGUGGCCUCCCGUCUC... 3'  
| | | | | | | |  
3' GAGGGAAGAAAGGAGGGCAGAA 5'  
| | |  
5'...UGUGGAGGGUGGCCUGGGCAGCC... 3'



### miR-139-5p and NDRG4 3'UTR interaction

Seed sequence position 1514-1522 in the  
**Wild type NDRG4-3'UTR**

**hsa-miR-139-5p**

**Mutant NDRG4-3'UTR**

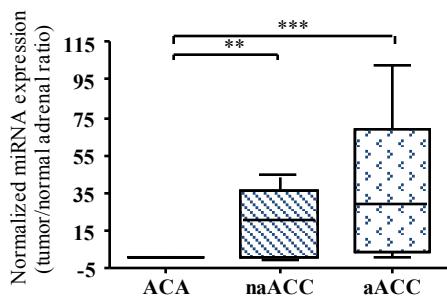
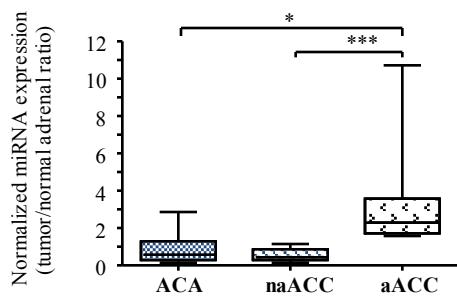
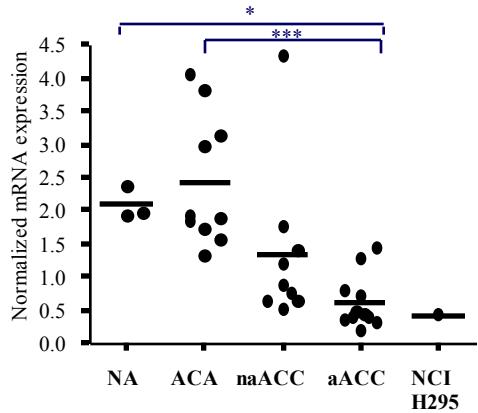
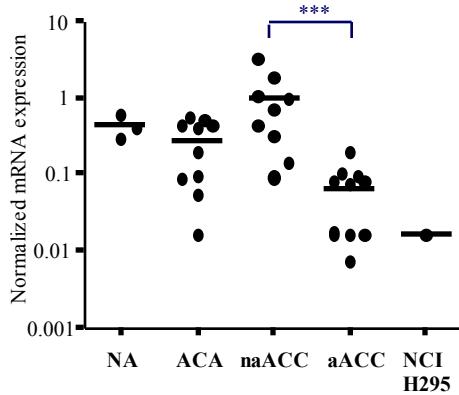
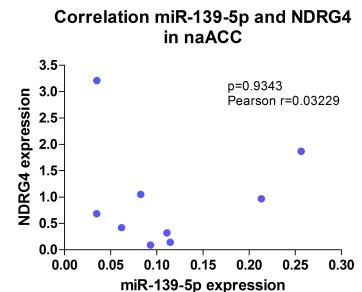
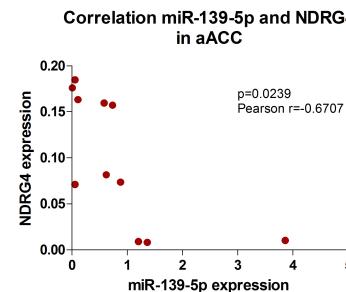
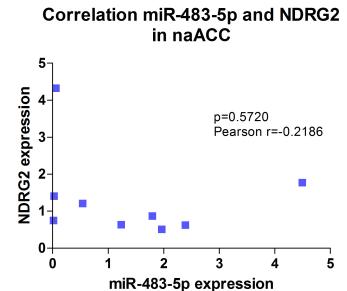
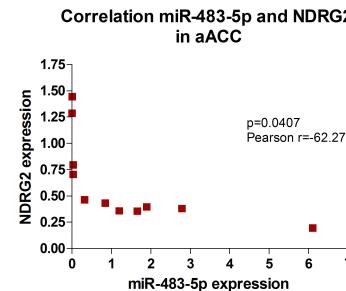
5'...GAAUUCAUCCCUCUUACUGUAGA... 3'  
| | | | | | | |  
3' GACCUCUGUGCACGUGACAUUCU 5'  
| |  
5'...GAAUUCAUCCCUCUUGCUCCCGCC... 3'

**Figure 1.** Schematic representations of miR-483-5p and miR-139-5p interaction with NDRG2 and NDRG4 respectively, according to prediction softwares.

**A**, miRNAs (in green) and mRNA target gene (in red) interaction and secondary structure using combined results from TargetScan, miRWalk and RNAHybrid prediction softwares.

**B**, NDRG2 or NDRG4 3'UTR pairing with miR-483-5p or miR-139-5p seed sequences, respectively. Nucleotides in red represent the mutations introduced in functional studies.

5'-UTR: 5'-untranslated region; CDS: coding sequence

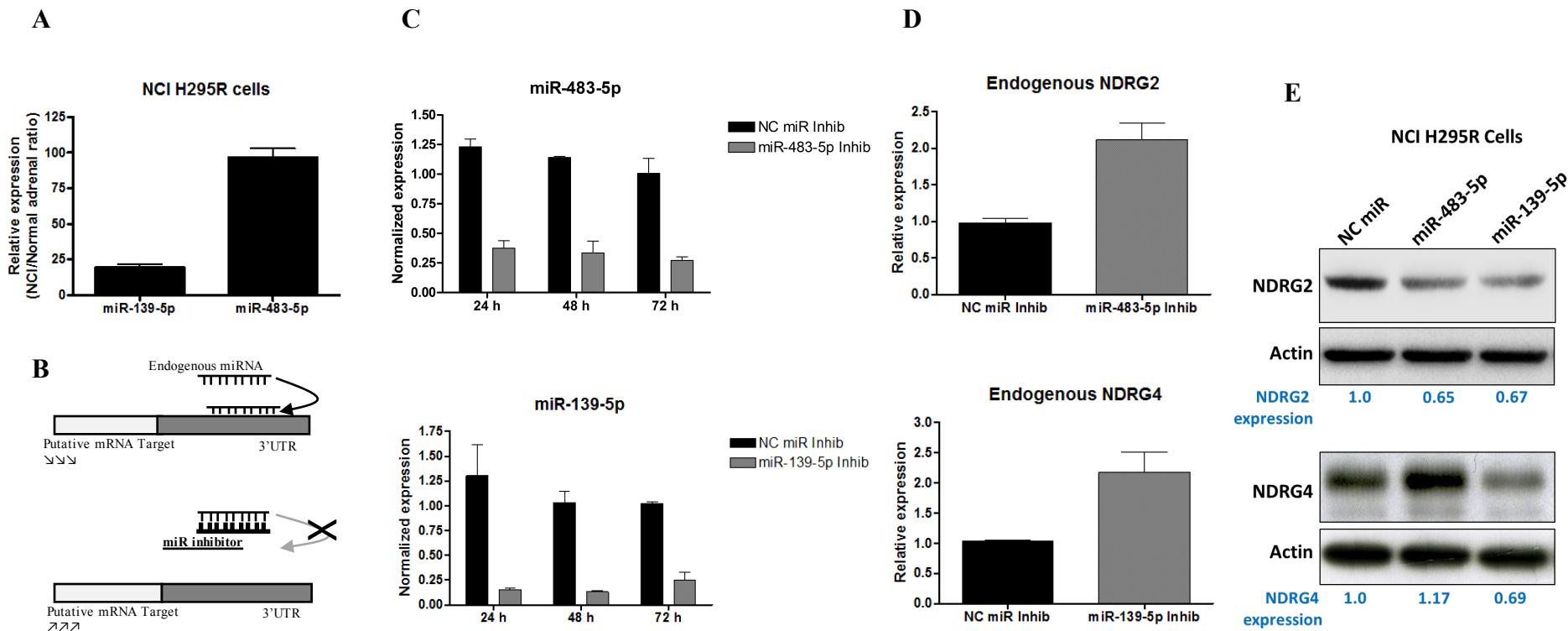
**A miR-483-5p****miR-139-5p****B NDRG2****NDRG4****C Expression level correlation (miR/target gene)****Figure 2. Inverse correlation between microRNAs expression and putative target gene expression in a cohort of human adrenocortical tissues.**

**A**, miRNA expression levels of miR-483-5p and miR-139-5p was measured by RT-qPCR in a cohort of human adrenocortical tissues from COMETE Network (10 adreno cortical adenomas (ACA), 9 non-aggressive adreno cortical carcinomas (naACC), 9 aggressive adrenocortical carcinomas (aACC), and 3 normal adreno cortical tissues (NA)). MiR-483-5p was up-regulated in ACC tumors compared with ACA, whereas miR-139-5p was up-regulated in aACC tumors compared with naACC.

**B**, NDRG2 and NDRG4 expression level was measured in the same cohort of patients by RT-qPCR. NDRG2 expression level was lower in aACC tissues compared with ACA and NA, whereas NDRG4 was under-expressed in aACC compared with naACC tissues.

**C**, Pearson correlation between expression levels of NDRG2 and miR-483-5p, and between expression levels of NDRG4 and miR-139-5p in aACC.

Statistical analysis was performed using Kruskal-Wallis test, \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ .



**Figure 3. Effect of miR-483-5p and miR-139-5p inhibitors on NDRG expression in NCI H295R cells.**

**A**, RT-qPCR analysis of endogenous microRNA expression level in NCI H295R cell line, normalized to RNU48 RNA and represented as a ratio of NCI H295R miRNA levels/normal adrenal miRNA levels. MiR-139-5p and miR-483-5p are overexpressed by about 100- and 20-fold, respectively, as compared to normal adrenocortical cells.

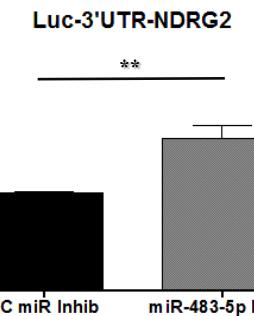
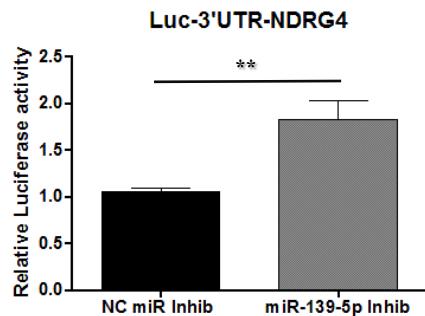
**B**, Schematic representation of miRNA inhibitor mechanism of action.

**C and D**, NCI H295R cells were transiently transfected for 24 h, 48 h or 72 h with miR-483-5p inhibitor, miR-139-5p inhibitor, or Negative Control miR inhibitor. **C**, MiRNA repression was checked by RT-qPCR. **D**, Effect of miR-inhibitors on endogenous NDRG2 and NDRG4 mRNA expression at 72h (n=3).

**E**, Western-Blot analysis of NDRG2 and NDRG4 protein expression in NCI H295R transfected with 100 nM and 50 nM of miR-483-5p or miR-139-5p mimics, respectively. NDRG2 or NDRG4 expressions were normalized to Actin. Shown is a representative experiment of 2 independent ones.

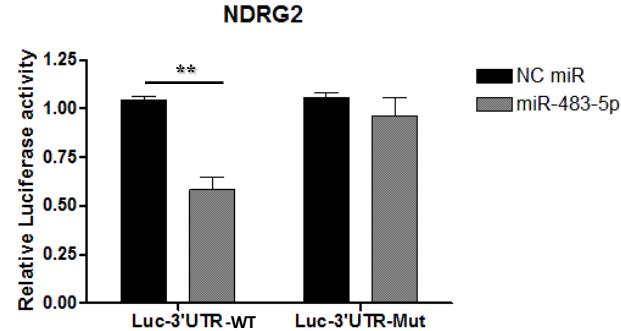
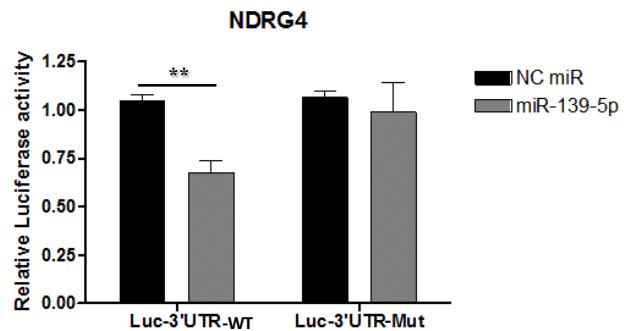
A

## NCI H295R Cells



B

## HEK293 Cells



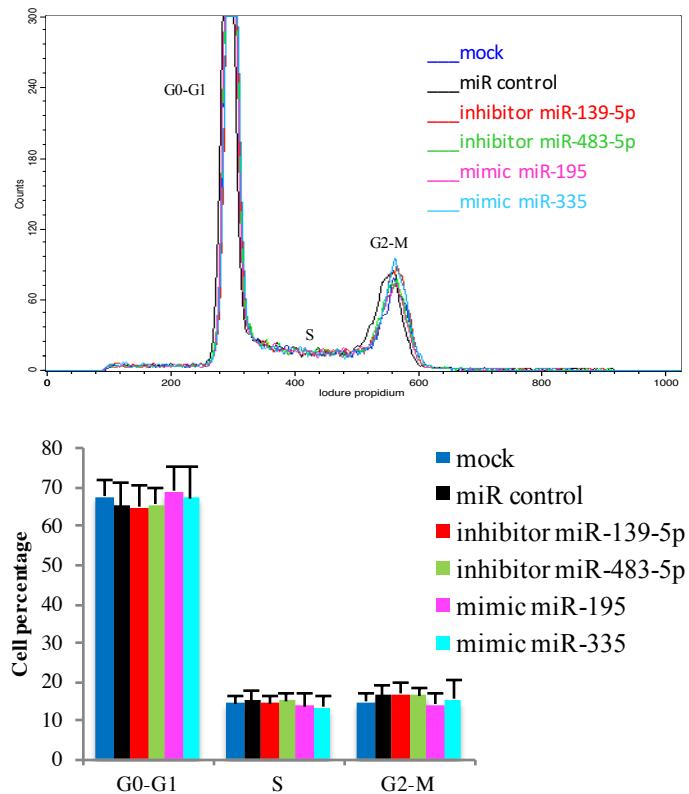
**Figure 4. MiR-483-5p and miR-139-5p functionally interact with NDRG2 and NDRG4 3'UTR.**

A, MiR-483-5p and miR-139-5p inhibition increases NDRG2-3'UTR or NDRG4-3'UTR-driven luciferase activity in NCI H295R cells.

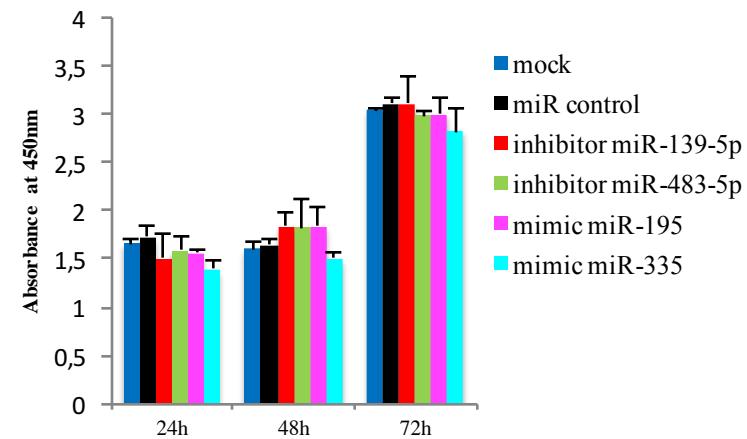
B, MiR-483-5p and miR-139-5p overexpression decreases NDRG2-3'UTR-Wild Type or NDRG4-3'UTR-Wild Type-driven luciferase activity in HEK293 cells. When mutations were introduced in the NDRG-3'UTR binding site, microRNAs overexpression in HEK293 cells had no effect on luciferase activity of pMIR-Luc-3'UTR-Mutant construct.

Represented are Firefly/Renilla luciferase activity ratios. Statistical analysis was performed using ANOVA, \*\* $p<0.01$ . Shown are the mean  $\pm$  S.E.M of 4 independent experiment performed in duplicates for wild type Luc-3'UTR. Luciferase activity of Luc-3'UTR Mut constructs represents Mean  $\pm$  SD from 2 experiments performed in duplicates.

### A NCI H295R cell cycle analysis : Flow cytometry



### B NCI H295R cell proliferation : WST1 assay

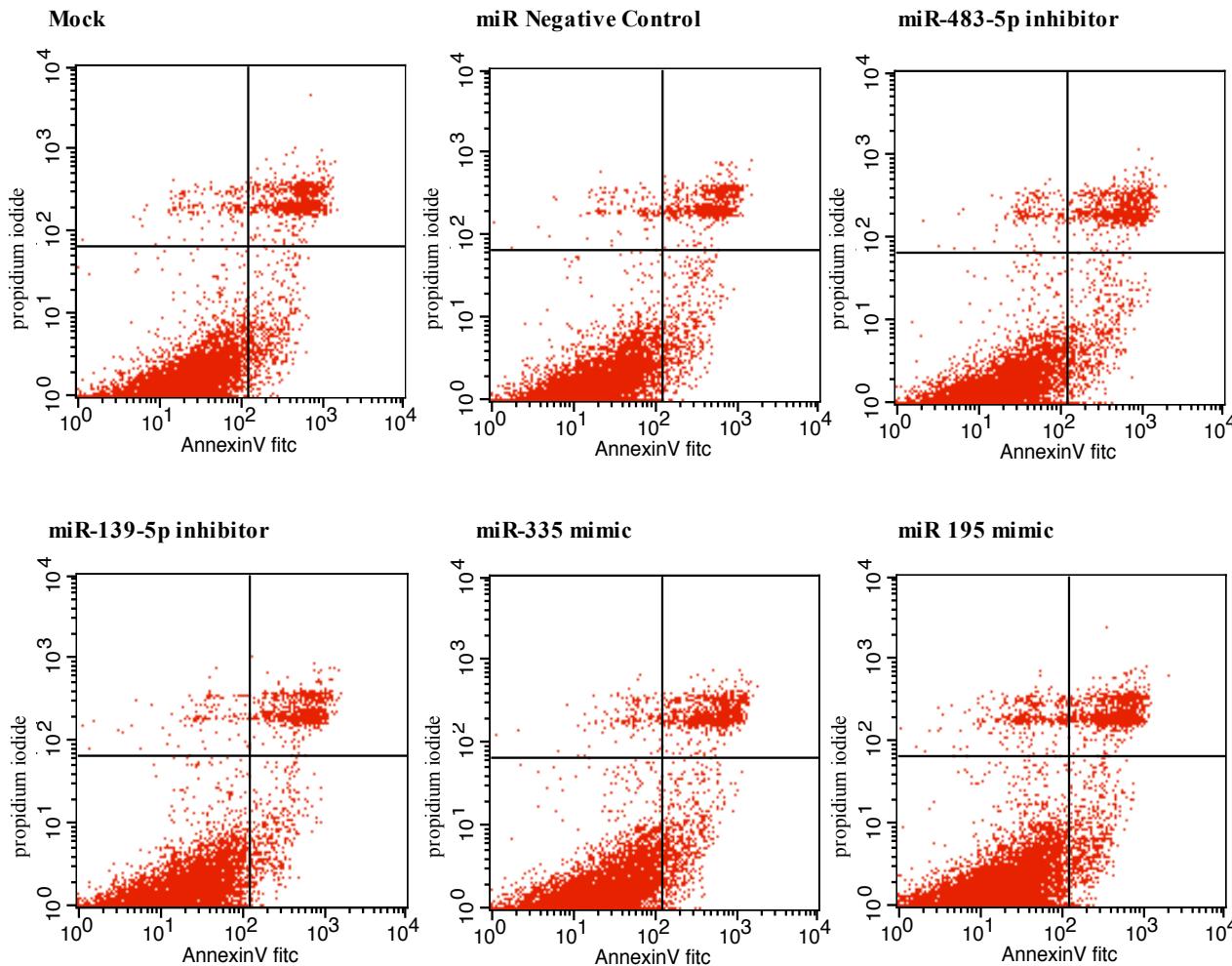


**Figure 5. Effect of miR-483-5p and miR-139-5p inhibition, or miR-195 and miR-335 overexpression, on cell cycle and cell proliferation in NCI-H295R cell line.**

MiRNA mimics (miR-195 and miR-335), miRNA inhibitors (miR-483-5p and miR-139-5p) or miR-negative control were transiently transfected in NCI-H295R cells.

**A,** Transfected and mock-transfected cells were stained with propidium iodide and analyzed by flow cytometry. The histogram shows the percentage of cells in the different cell cycle phases determined in each condition of transfection. No significant differences in cell cycle phases were observed between each condition (n=2).

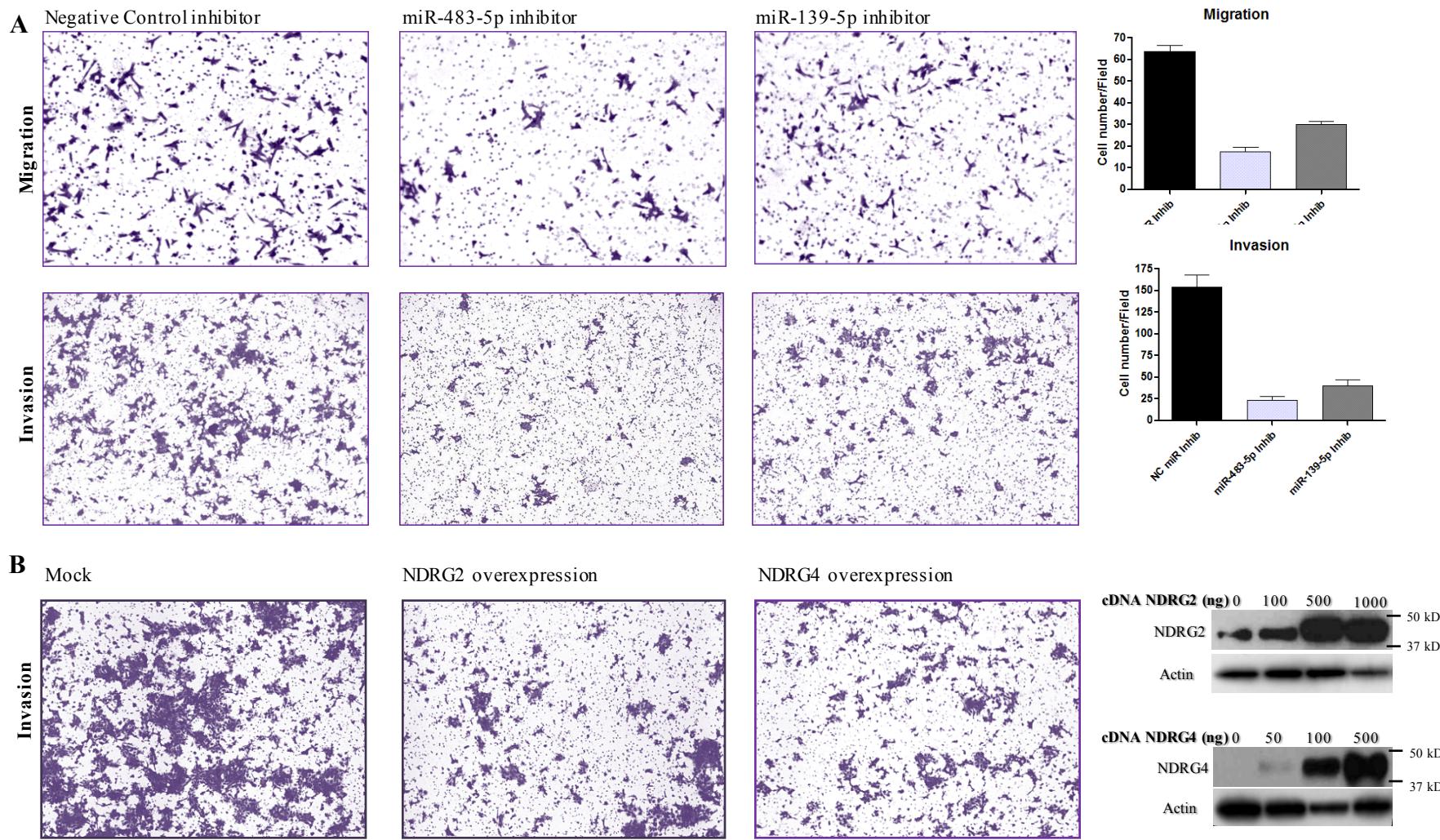
**B,** Proliferation of NCI-H295 cells was measured by WST1 assays. 24 h, 48h and 72 h after transfection, WST1 was added in culture wells and formazan products were detected by colorimetric method reflecting cell viability. No statistical differences in cell proliferation rate were observed between each condition (n=4).



**Figure 6. Effect of miR-483-5p and miR-139-5p inhibition, or miR-195 and miR-335 overexpression, on NCI-H295R cell apoptosis.**

Cell apoptosis was assessed by Annexin V-FITC assay. MiRNA mimics (miR-195 and miR-335), miRNA inhibitors (miR-483-5p and miR-139-5p) or miR-negative control were transiently transfected in NCI-H295R cells. Transfected and mock-transfected cells were stained with Annexin V-FITC and propidium iodide (PI), and were analyzed by flow cytometry. The lower square on the right represent cells stained by Annexin V but not by PI (Annexin V+/PI-), reflecting cells in early stage of apoptosis. The upper square on the right reflects cells in late apoptosis and those undergoing necrosis or damaged cells (Annexin V+/PI+).

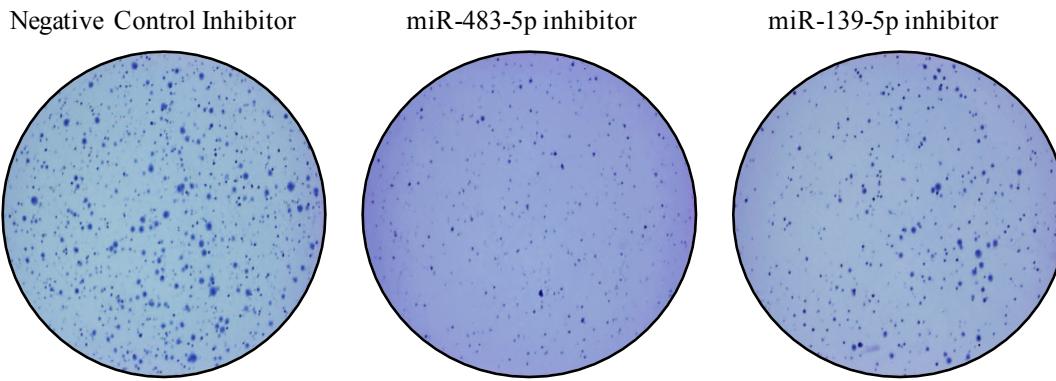
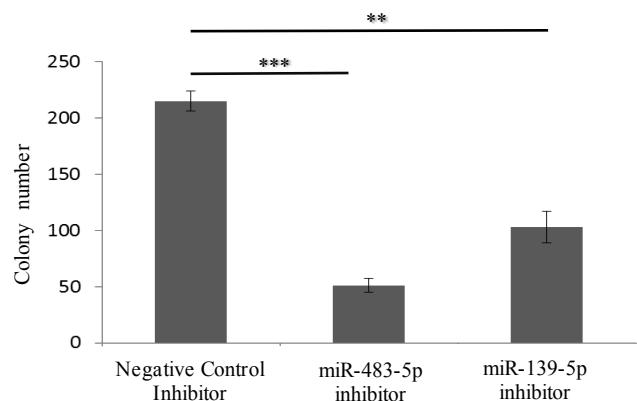
*No significant changes in the percentage of cells undergoing early apoptosis were observed between each condition (the rate of cells labeled Annexin V+/PI+ were the same in each culture condition). One representative of 2 independent experiments is shown (n=2).*



**Figure 7. Effect of miR-483-5p and miR-139-5p inhibition, or NDRG target gene overexpression on migration and invasion capacities of NCI-H295R cells.**

*Transwell assays were performed as described in Material and Methods to assess cell migration and invasion.*

**A**, NCI H295R cells were transiently transfected with negative control miR, miR-483-5p or miR-139-5p inhibitors, then analyzed in migration and invasion assays. The right panels are quantification of cell number in five random fields per well (mean  $\pm$  SEM) and are representative of four independent experiments. **B**, Mock-transfected, NDRG2- or NDRG4-transfected NCI H295R cells (500 ng of plasmid) were analyzed in invasion assay. Western Blots on the right represent a dose-response for NDRG plasmids in NCI-H295R cells.

**A****B**

**Figure 8. Effect of miR-483-5p and miR-139-5p inhibition on anchorage-independent growth of NCI-H295R cells.**

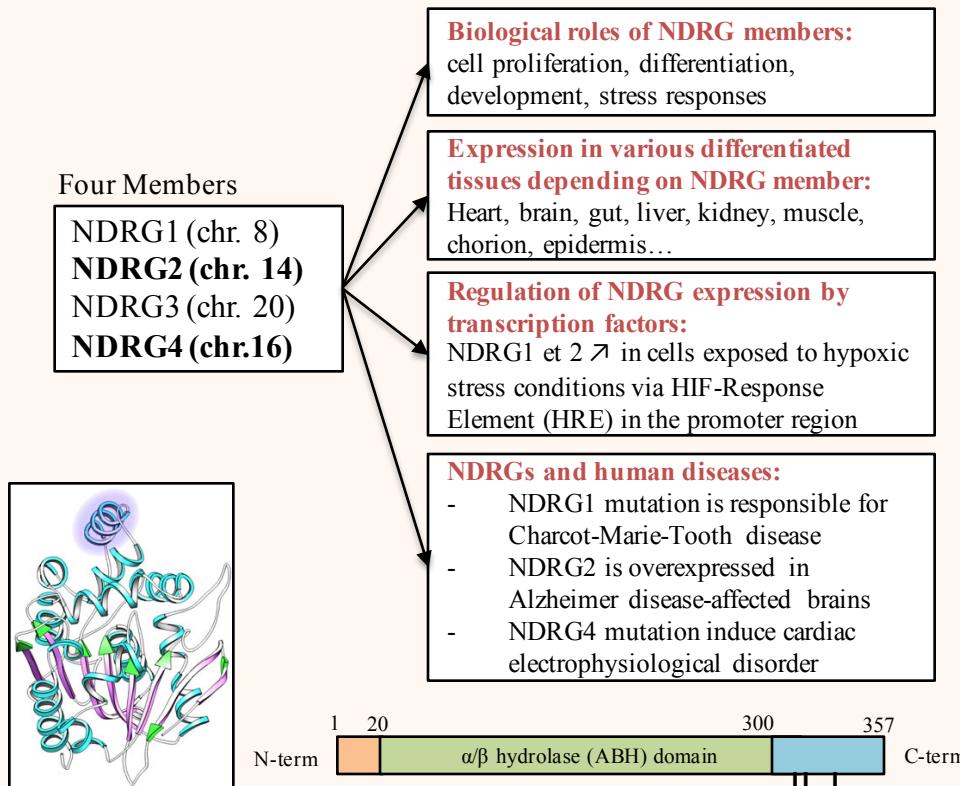
Soft agar colony formation assay was performed as described in Materiel and Methods.

**A,** The colony size was greater in miR-Negative Control-transfected cells as compared to miR-inhibitors-transfected cells which also display lower colony-forming ability.

**B,** Quantification of colony number in 3 independent experiments.

\*\* p<0.01, \*\*\* p<0.001.

## N-Myc Downstream-Regulated gene (NDRG) family



## Human NDRG2 structure

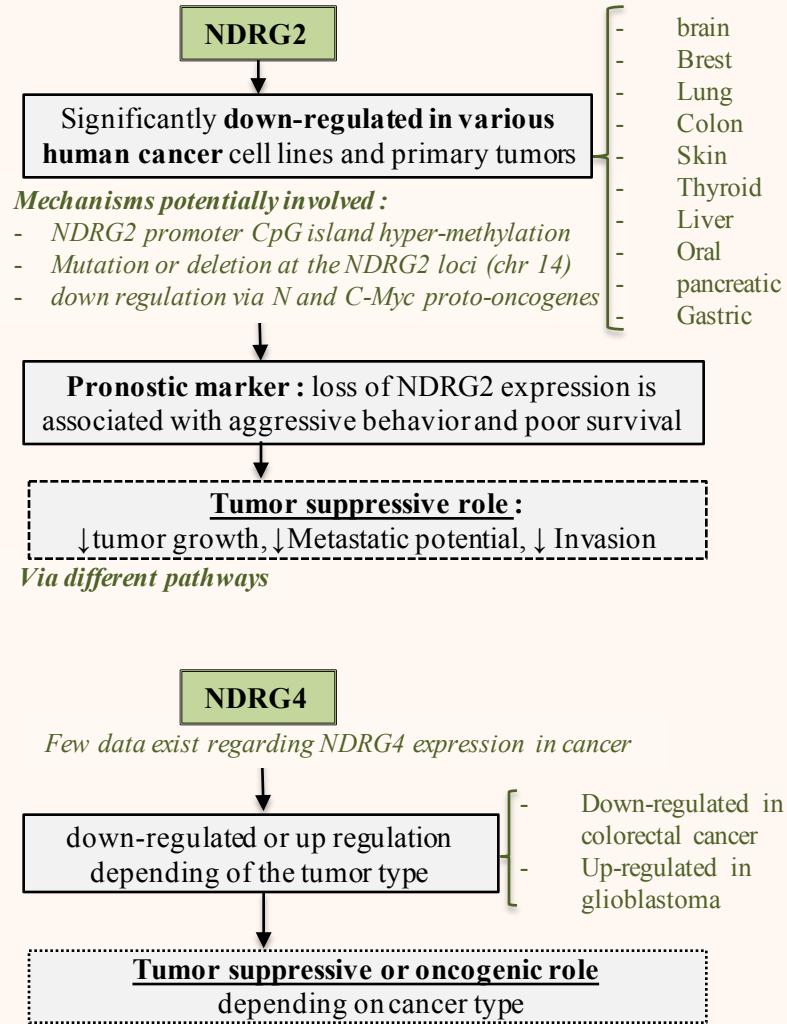
Helix  $\alpha$ 6 (circled in blue in the figure) in the protein cap, is involved in molecular interactions with  $\beta$ -catenin signaling.

## NDRG family protein structure

On the basis of their sequences, they resemble to the  $\alpha/\beta$  hydrolase (ABH) superfamily, but they have no enzymatic activity

- 4 members share 53–65% amino acid sequence identity
  - Most variation are located in the N-and C-terminal regions
  - The ABH domain shows the highest homology

## NDRG2 and NDRG4 in cancer



**Figure 9. The multiple functions of the N-Myc Downstream-Regulated gene (NDRG) family.**

The NDRG family is highly conserved between species. As they are repressed by the Myc oncogene, they have been argued to have tumor-suppressive and anti-metastatic functions. The NDRGs may play important roles in the development of cancer and other diseases. The molecular mechanisms underlying their diverse actions await further investigations.

THÈSE SOUTENUE PAR : Claire AGOSTA

TITRE : **MiR-483-5p et miR-139-5p contribuent à l'invasivité et la tumorigénérité du carcinome corticosurrénalien**

CONCLUSION :

Le carcinome corticosurrénalien (CCS) est un cancer rare et très agressif avec un taux de survie à 5 ans de 38%, dépendant du stade tumoral. Le seul traitement curatif repose sur une exérèse chirurgicale complète de la tumeur primitive mais le traitement des formes avancées est limité et souvent inefficace. Des efforts nationaux, en collaboration internationale, sont actuellement menés pour identifier les mécanismes moléculaires et les altérations génétiques à l'origine de la carcinogénèse corticosurrénaliennes afin de mettre en place des outils diagnostiques, pronostiques et thérapeutiques. Les microARNs sont des petits ARNs non codants qui régulent l'expression de nombreux gènes impliqués dans les processus cellulaires clefs. Leur rôle dans le domaine de la cancérologie est maintenant bien connu. L'analyse du profil d'expression des microARNs dans les tumeurs corticosurrénaliennes a montré une forte dérégulation de leur niveau d'expression dans les CCS par comparaison aux adénomes corticosurrénaliens. En effet, notre équipe a montré avec d'autres que miR-483-5p est le microARN le plus significativement surexprimé au niveau tumoral dans les CCS. De façon intéressante ce microARN est également détecté dans le sérum de patients atteints de CCA agressif et pourrait donc être utilisé comme biomarqueur pronostic. MiR-139-5p est également fortement surexprimé dans les CCS agressifs (récidivants ou au stade métastatique) comparés aux CCS de bon pronostic.

Dans notre étude, nous avons utilisé une lignée cellulaire de carcinome corticosurrénalien humain agressif pour étudier le rôle fonctionnel de miR-483-5p et miR-139-5p dans la tumorigénèse corticosurrénaliennes en identifiant leurs gènes cibles et en analysant leur impact sur les processus cellulaires clefs tel que la prolifération cellulaire, l'apoptose, la migration, et l'invasion.

La prédiction des cibles a été réalisée en utilisant les bases de données miRWALK et TargetScan, combinées avec le profil d'expression génique des tumeurs corticosurrénaliennes, publié précédemment (de Reyniès et al. 2009). Le niveau d'expression tumoral de miR-483-5p et miR-139-5p a été mesuré par RT-qPCR dans une cohorte de 28 patients issus de la banque du réseau national COMETE (COrtico- MEduollo Tumeurs Endocrines), et corrélé au niveau d'expression

des gènes cibles hypothétiques. La région 3'-non traduite (3'UTR) des gènes cibles a été clonée en aval d'un gène rapporteur Luciferase pour évaluer l'interaction microARN-ARN messager. Le rôle des deux microARNs dans le phénotype malin de la lignée cellulaire NCI-H295R a été étudié par transfection d'inhibiteurs de microARNs.

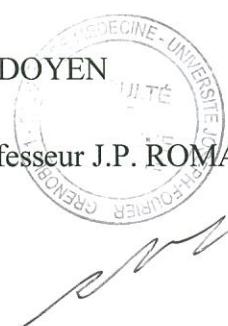
Nous avons identifié NDRG2 (N-Myc Downstream-Regulated Gene 2) et NDRG4 comme cibles de miR-483-5p et miR-139-5p, respectivement. L'expression de NDRG2 et NDRG4 dans les CCS était inversement corrélée à l'expression de miR-483-5p et miR-139-5p, respectivement. L'inhibition de miR-483-5p et miR-139-5p dans les cellules NCI-H295R entraîne une augmentation du niveau d'ARNm de NDRG2 et NDRG4. De plus, les transcrits de NDRG2 et NDRG4 sont régulés négativement via un site d'interaction spécifique avec les microARNs. Enfin, l'inhibition de miR-483-5p et de miR-139-5p n'a pas affecté la prolifération ou l'apoptose des cellules NCI-H295R, mais a inhibé leur caractère invasif et leur croissance en gel d'agar sans ancrage cellulaire.

Nos données suggèrent que miR-483-5p et miR-139-5p contribuent à l'agressivité des CCS en réprimant l'expression de deux gènes suppresseurs de tumeurs, NDRG2 et NDRG4. Les voies de signalisation et les mécanismes moléculaires affectées par les microARNs et leurs gènes cibles doivent être approfondis. L'utilisation des miR-483-5p et miR-139-5p comme cibles thérapeutiques directe ou indirecte semble être prometteuse pour limiter le caractère invasif des carcinomes corticosurrénaliens.

#### VU ET PERMIS D'IMPRIMER

Grenoble, le 22/02/2015

LE DOYEN



Professeur J.P. ROMANET

LE PRESIDENT DE LA THÈSE

Professeur O. CHABRE

Pr O. CHABRE  
CHU de Grenoble - CS10217  
2<sup>e</sup> étage - Pav les Ecrins  
4 rue du Musée  
38043 GRENOBLE CEDEX 9  
Tél : 04.76.76.56.80

# INTÉGRATION DE LA COLLECTION DE TUMEURS SURRÉNALIENNES DANS LE CENTRE DE RESSOURCES BIOLOGIQUES (CRB) DU CHU DE GRENOBLE

Durant mon internat, j'ai participé à l'homologation de la banque de tumeurs surrénales du Département d'Anatomie et Cytologie Pathologiques (Collection DACP déclarée au ministère de l'enseignement supérieur et de la recherche DC-2008-584 collection 1) afin de permettre son intégration dans le Centre de Ressources Biologiques (CRB) du CHU de Grenoble.

Une telle banque tissulaire représente un outil de travail précieux et reconnu par la communauté scientifique pour la recherche clinique et fondamentale, et permet d'élaborer des collaborations locorégionales, nationales (réseau national COMETE\_COrtico- et MEdullo- Tumeurs Endocrines) et internationales (réseau Européen ENS@t\_ The European Network for the Study of Adrenal Tumors), dans un cadre règlementé.

Le CRB a pour vocation d'accueillir des prélèvements biologiques de patients sur la thématique "CANCER" dans le cadre de la loi de Bioéthique (Loi n° 2004-800 du 6 août 2004) et de conserver ces ressources biologiques à des fins diagnostiques ou de recherche. Ces activités ont fait l'objet d'une demande d'autorisation accordée par le Ministère de la Recherche, d'une validation auprès du Comité de Protection des Personnes local (CPP IV).

Entre 1990 et fin 2012, 316 échantillons de tumeurs surrénales ont été cryo-conservés à -80°C et stockés au sein du Département d'Anatomie et Cytologie Pathologiques (DACP\_ Institut de Biologie et de Pathologie du CHU de Grenoble). Ces tumeurs ont été prélevées dans le cadre des soins de patients au bloc opératoire du CHU de Grenoble (service de chirurgie thoracique, vasculaire et endocrinienne), à des fins thérapeutique et diagnostique.

Les informations anatomo-pathologiques pour l'ensemble des 316 prélèvements ont été collectées à partir de novembre 2012 au DACP sous la responsabilité du Dr Nathalie STURM et avec l'accord du CRB sous la direction du Pr MOSSUZ Pascal (**Tableaux 1 et 2**). La majorité des prélèvements concernent des tumeurs d'origine cortico ou médullo-surrénaliennes. Les données anatomo-pathologiques (score de Weiss, classification ENS@T 2008 des carcinomes corticosurrénaliens

(CCS), caractéristiques histologiques) et les données biocliniques (type de sécrétion hormonale, invasivité loco-régionale et à distance des CCS et évolution clinique des patients) ont été recueillies pour l'ensemble des adénomes et carcinomes cortico-surrénaux, en collaboration avec le Pr Olivier CHABRE du service d'endocrinologie et le Pr Philippe CHAFFANJON du service de chirurgie endocrinienne du CHU de Grenoble. La conservation des prélèvements au CRB et leur cession à la recherche scientifique nécessite une information du patient. La non-opposition du patient sous forme d'un consentement signé doit être recueillie du vivant du patient et lorsque la tumeur a été prélevée après le Décret du 10 août 2007 *relatif au prélèvement, à la conservation et à la préparation à des fins scientifiques d'éléments du corps humain (tissus ou cellules)*.

|                                                    |                                                                                                    | Total |    |    |
|----------------------------------------------------|----------------------------------------------------------------------------------------------------|-------|----|----|
| <b>Tumeurs d'origine Corticosurrénalienne (CS)</b> | Adénomes                                                                                           | 137   |    |    |
|                                                    | Carcinomes                                                                                         | 30    |    |    |
|                                                    | Hyperplasies                                                                                       | 30    |    |    |
|                                                    | Tumeurs secondaires de carcinome CS                                                                | 3     |    |    |
| <b>Tumeurs sécrétant des Catécholamines</b>        | Phéochromocytomes                                                                                  | 58    | 66 | 73 |
|                                                    | Phéochromocytomes composites (ganglioneurome, cellules de Schwann ou ganglioneuroblastome associé) | 8     |    |    |
|                                                    | Paragangliomes                                                                                     | 6     | 7  |    |
|                                                    | Paragangliome composite                                                                            | 1     |    |    |
|                                                    |                                                                                                    |       |    |    |
| <b>Autres tumeurs surrénales</b>                   | Ganglioneuromes                                                                                    | 7     |    | 38 |
|                                                    | Schwannomes                                                                                        | 3     |    |    |
|                                                    | Neuroblastome                                                                                      | 1     |    |    |
|                                                    | Lymphangiome                                                                                       | 1     |    |    |
|                                                    | Angiome                                                                                            | 1     |    |    |
|                                                    | Myélolipomes                                                                                       | 4     |    |    |
| <b>Tumeurs intra surrénales d'autre origine</b>    | Métastases de primitif extra surrénalien                                                           | 16    |    |    |
|                                                    | Lymphomes malins non Hodgkinien                                                                    | 3     |    |    |
|                                                    | Abcès                                                                                              | 1     |    |    |
|                                                    | Kyste embryonnaire mésodermique                                                                    | 1     |    |    |
|                                                    |                                                                                                    |       |    |    |
| <b>Surrénale normale</b>                           |                                                                                                    | 5     |    | 5  |

**Tableau 1. 316 prélèvements tissulaires issus de la bio-banque initiale de tumeurs surrénales et stockés au DACP entre 1990 et 2012, avant intégration dans la collection de tumeurs surrénales du CRB en 2013.**

|           |                                  | Corticosurrénale (CS) |            |                                         |
|-----------|----------------------------------|-----------------------|------------|-----------------------------------------|
|           |                                  | Adénomes              | Carcinomes | Hyperplasies                            |
| Sécrétion | Aldostérone                      | 56                    |            | 3                                       |
|           | Cortisol                         | 32                    | 9          | ACTH dépendant 10<br>ACTH indépendant 6 |
|           | Cortisol + androgènes            |                       | 8          |                                         |
|           | Cortisol + oestrogènes           |                       | 1          |                                         |
|           | Cortisol + DOC                   |                       | 1          |                                         |
|           | Oestrogènes                      | 1                     |            |                                         |
|           | Non fonctionnel                  | 17                    | 8          |                                         |
|           | Déficit 21 hydroxylase           |                       |            | 2                                       |
|           | Indéterminé (données manquantes) | 31                    | 3          | 9                                       |
| Total     |                                  | 137                   | 30         | 30                                      |

**Tableau 2. Tumeurs d'origine corticosurrénalienne issues de la bio-banque initiale (stockées au DACP entre 1990 et 2012), avec le type de sécrétion hormonale.**

Ce travail nous a permis de sélectionner et **d'intégrer de façon rétrospective 64 tumeurs corticosurrénaliennes** (prélevées entre août 1994 et octobre 2012) au sein du CRB de Grenoble :

- 28 adénomes corticosurrénaliens (sécrétant du cortisol).
- 3 hyperplasies macro-nodulaires corticosurrénaliennes (sécrétant du cortisol).
- 30 carcinomes corticosurrénaliens (dont 2 carcino-sarcomes, et 2 carcinomes corticosurrénaliens à cellules oncocytaires). La totalité des carcinomes a été inclus dans la banque CRB.
- 3 métastases de carcinome corticosurrénalien (localisation pulmonaire, veine cave inférieure, récidive locale sous diaphragmatique).

Depuis mars 2014, **25 tumeurs surrénales primitives supplémentaires, d'origine à la fois cortico- et médullo-surrénalienne, ont été intégrées de façon prospective** dans le CRB :

- 15 adénomes corticosurrénaliens (dont 2 adénomes à cellules oncocytaires)
- 2 carcinomes corticosurrénaliens (dont 1 carcinome à cellules oncocytaires)
- 1 hyperplasie macro-nodulaire corticosurrénalienne
- 7 phéochromocytomes

Les objectifs futurs pour enrichir la bio-banque de tumeurs surrénales sera d'associer régulièrement les données anatomo-cliniques aux prélèvements congelés et stockés de façon prospective dans le CRB. La cession des prélèvements tumoraux à la recherche à déjà fait l'objet de partenariat entre le CRB et la communauté scientifique, avec notamment l'envoi de 14 échantillons de carcinomes corticosurrénaux au réseau national COMETE en 2014. D'autres projets de partenariats sont en cours afin de valoriser cette banque de tumeurs rares. Nous avons également comme objectif de rendre ce matériel accessible par la base européenne ENS@T, ce qui supposera d'harmoniser les données anatomo-cliniques à enregistrer dans la banque.

Le recueil d'un document d'information signé par le patient pour l'utilisation d'un échantillon tumoral à des fins de recherche est indispensable et devra être associé systématiquement à chaque prélèvement inclus dans le CRB, ce qui n'est pas encore effectif actuellement. L'améliorer du trajet des consentements signés depuis la consultation préopératoire, jusqu'à la réception du prélèvement au niveau du CRB, nécessite une collaboration entre le service d'endocrinologie, de chirurgie endocrinienne, le DAPC et le CRB. La rédaction d'un nouveau document d'information des patients à destination du CRB est en cours de validation.

Enfin, parmi les perspectives futures, la conservation de prélèvements sanguins de patients dans les périodes pré- et post-opératoires, permettra d'enrichir cette bio-banque afin d'obtenir une sérothèque et DNAthèque associées aux fragments tumoraux. Ces données seront particulièrement intéressantes dans le cas des cancers surrénaux afin de définir de nouveaux outils diagnostique, pronostique et thérapeutique.

Les responsables de la COLLECTION SURRENALE au sein du CRB sont le **Dr Nathalie STURM** et le **Pr Olivier CHABRE** (Responsables Médicaux), le **Pr Philippe CHAFFANJON** (Responsable de l'équipe chirurgicale). Le CRB de Grenoble est sous la direction du **Pr Pascal MOSSUZ**. Les correspondants au niveau du CRB sont **monsieur Philippe LORIMIER** (Ingénieur Biogiste, Responsable opérationnel), **madame Florence IMBURCHIA** (Ingénieur Qualité), et **monsieur Gabriel SCHUMMER** (Responsables des données/ARC).

*CRB du CHU de Grenoble  
INSTITUT DE BIOLOGIE ET PATHOLOGIE  
Boulevard de la Chantourne  
RDC Bas, BP217 X 38043 Grenoble cedex 09*

Doyen de la Faculté : **M. le Pr. Jean Paul ROMANET**

**Année 2014-2015**

**ENSEIGNANTS A L'UFR DE MEDECINE**

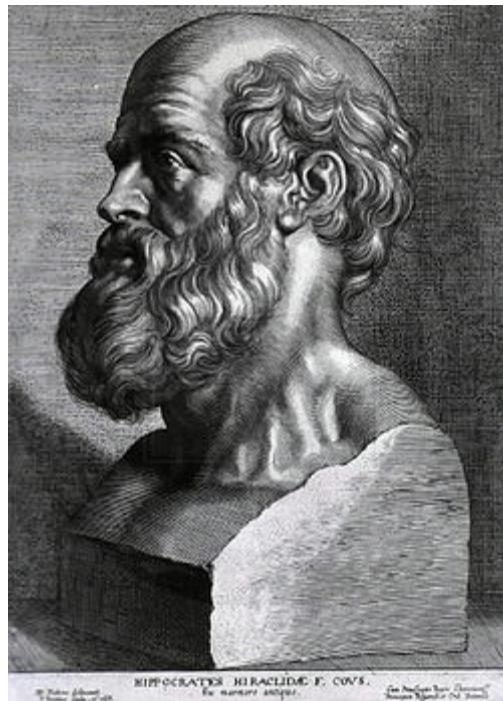
| CORPS  | NOM-PRENOM                    | Discipline universitaire                                             |
|--------|-------------------------------|----------------------------------------------------------------------|
| PU-PH  | ALBALADEJO Pierre             | Anesthésiologie réanimation                                          |
| PU-PH  | APTEL Florent                 | Ophthalmologie                                                       |
| PU-PH  | ARVIEUX-BARTHELEMY Catherine  | chirurgie générale                                                   |
| PU-PH  | BALOSSO Jacques               | Radiothérapie                                                        |
| PU-PH  | BARRET Luc                    | Médecine légale et droit de la santé                                 |
| PU-PH  | BENHAMOU Pierre Yves          | Endocrinologie, diabète et maladies métaboliques                     |
| PU-PH  | BERGER François               | Biologie cellulaire                                                  |
| PU-PH  | BETTEGA Georges               | Chirurgie maxillo-faciale, stomatologie                              |
| MCU-PH | BIDART-COUTTON Marie          | Biologie cellulaire                                                  |
| MCU-PH | BOISSET Sandrine              | Agents infectieux                                                    |
| PU-PH  | BONAZ Bruno                   | Gastro-entérologie, hépatologie, addictologie                        |
| MCU-PH | BONNETERRE Vincent            | Médecine et santé au travail                                         |
| PU-PH  | BOSSON Jean-Luc               | Biostatiques, informatique médicale et technologies de communication |
| MCU-PH | BOTTARI Serge                 | Biologie cellulaire                                                  |
| PU-PH  | BOUGEROL Thierry              | Psychiatrie d'adultes                                                |
| PU-PH  | BOUILLET Laurence             | Médecine interne                                                     |
| MCU-PH | BOUZAT Pierre                 | Réanimation                                                          |
| PU-PH  | BRAMBILLA Christian           | Pneumologie                                                          |
| PU-PH  | BRAMBILLA Elisabeth           | Anatomie et cytologie pathologiques                                  |
| MCU-PH | BRENIER-PINCHART Marie Pierre | Parasitologie et mycologie                                           |
| PU-PH  | BRICAULT Ivan                 | Radiologie et imagerie médicale                                      |
| PU-PH  | BRICHON Pierre-Yves           | Chirurgie thoracique et cardio- vasculaire                           |
| MCU-PH | BRIOT Raphaël                 | Thérapeutique, médecine d'urgence                                    |
| PU-PH  | CAHN Jean-Yves                | Hématologie                                                          |
| MCU-PH | CALLANAN-WILSON Mary          | Hématologie, transfusion                                             |
| PU-PH  | CARPENTIER Françoise          | Thérapeutique, médecine d'urgence                                    |
| PU-PH  | CARPENTIER Patrick            | Chirurgie vasculaire, médecine vasculaire                            |
| PU-PH  | CESBRON Jean-Yves             | Immunologie                                                          |
| PU-PH  | CHABARDES Stephan             | Neurochirurgie                                                       |

|        |                                |                                                                         |
|--------|--------------------------------|-------------------------------------------------------------------------|
| PU-PH  | <b>CHABRE Olivier</b>          | Endocrinologie, diabète et maladies métaboliques                        |
| PU-PH  | <b>CHAFFANJON Philippe</b>     | Anatomie                                                                |
| PU-PH  | <b>CHAVANON Olivier</b>        | Chirurgie thoracique et cardio- vasculaire                              |
| PU-PH  | <b>CHIQUET Christophe</b>      | Ophthalmologie                                                          |
| PU-PH  | <b>CINQUIN Philippe</b>        | Biostatistiques, informatique médicale et technologies de communication |
| PU-PH  | <b>COHEN Olivier</b>           | Biostatistiques, informatique médicale et technologies de communication |
| PU-PH  | <b>COUTURIER Pascal</b>        | Gériatrie et biologie du vieillissement                                 |
| PU-PH  | <b>CRACOWSKI Jean-Luc</b>      | Pharmacologie fondamentale, pharmacologie clinique                      |
| PU-PH  | <b>DE GAUDEMARIS Régis</b>     | Médecine et santé au travail                                            |
| PU-PH  | <b>DEBILLON Thierry</b>        | Pédiatrie                                                               |
| MCU-PH | <b>DECAENS Thomas</b>          | Gastro-entérologie, Hépatologie                                         |
| PU-PH  | <b>DEMATTÉIS Maurice</b>       | Addictologie                                                            |
| PU-PH  | <b>DEMONGEOT Jacques</b>       | Biostatistiques, informatique médicale et technologies de communication |
| MCU-PH | <b>DERANSART Colin</b>         | Physiologie                                                             |
| PU-PH  | <b>DESCOTES Jean-Luc</b>       | Urologie                                                                |
| MCU-PH | <b>DETANTE Olivier</b>         | Neurologie                                                              |
| MCU-PH | <b>DIETERICH Klaus</b>         | Génétique et procréation                                                |
| MCU-PH | <b>DOUTRELEAU Stéphane</b>     | Physiologie                                                             |
| MCU-PH | <b>DUMESTRE-PERARD Chantal</b> | Immunologie                                                             |
| PU-PH  | <b>EPAULARD Olivier</b>        | Maladies Infectieuses et Tropicales                                     |
| PU-PH  | <b>ESTEVE François</b>         | Biophysique et médecine nucléaire                                       |
| MCU-PH | <b>EYSSERIC Hélène</b>         | Médecine légale et droit de la santé                                    |
| PU-PH  | <b>FAGRET Daniel</b>           | Biophysique et médecine nucléaire                                       |
| PU-PH  | <b>FAUCHERON Jean-Luc</b>      | chirurgie générale                                                      |
| MCU-PH | <b>FAURE Julien</b>            | Biochimie et biologie moléculaire                                       |
| PU-PH  | <b>FERRETTI Gilbert</b>        | Radiologie et imagerie médicale                                         |
| PU-PH  | <b>FEUERSTEIN Claude</b>       | Physiologie                                                             |
| PU-PH  | <b>FONTAINE Éric</b>           | Nutrition                                                               |
| PU-PH  | <b>FRANCOIS Patrice</b>        | Epidémiologie, économie de la santé et prévention                       |
| PU-PH  | <b>GARBAN Frédéric</b>         | Hématologie, transfusion                                                |
| PU-PH  | <b>GAUDIN Philippe</b>         | Rhumatologie                                                            |
| PU-PH  | <b>GAVAZZI Gaétan</b>          | Gériatrie et biologie du vieillissement                                 |
| PU-PH  | <b>GAY Emmanuel</b>            | Neurochirurgie                                                          |
| MCU-PH | <b>GILLOIS Pierre</b>          | Biostatistiques, informatique médicale et technologies de communication |
| PU-PH  | <b>GODFRAIND Catherine</b>     | Anatomie et cytologie pathologiques (type clinique)                     |
| MCU-PH | <b>GRAND Sylvie</b>            | Radiologie et imagerie médicale                                         |
| PU-PH  | <b>GRIFFET Jacques</b>         | Chirurgie infantile                                                     |
| MCU-PH | <b>GUZUN Rita</b>              | Endocrinologie, diabétologie, nutrition, éducation thérapeutique        |
| PU-PH  | <b>HALIMI Serge</b>            | Nutrition                                                               |
| PU-PH  | <b>HENNEBICQ Sylviane</b>      | Génétique et procréation                                                |

|        |                                             |                                                                      |
|--------|---------------------------------------------|----------------------------------------------------------------------|
| PU-PH  | <b>HOFFMANN Pascale</b>                     | Gynécologie obstétrique                                              |
| PU-PH  | <b>HOMMEL Marc</b>                          | Neurologie                                                           |
| PU-PH  | <b>JOUK Pierre-Simon</b>                    | Génétique                                                            |
| PU-PH  | <b>JUVIN Robert</b>                         | Rhumatologie                                                         |
| PU-PH  | <b>KAHANE Philippe</b>                      | Physiologie                                                          |
| PU-PH  | <b>KRACK Paul</b>                           | Neurologie                                                           |
| PU-PH  | <b>KRAINIK Alexandre</b>                    | Radiologie et imagerie médicale                                      |
| PU-PH  | <b>LABARERE José</b>                        | Epidémiologie ; Eco. de la Santé                                     |
| PU-PH  | <b>LANTUEJOUL Sylvie</b>                    | Anatomie et cytologie pathologiques                                  |
| MCU-PH | <b>LAPORTE François</b>                     | Biochimie et biologie moléculaire                                    |
| MCU-PH | <b>LARDY Bernard</b>                        | Biochimie et biologie moléculaire                                    |
| MCU-PH | <b>LARRAT Sylvie</b>                        | Bactériologie, virologie                                             |
| MCU-PH | <b>LAUNOIS-ROLLINAT Sandrine</b>            | Physiologie                                                          |
| PU-PH  | <b>LECCIA Marie-Thérèse</b>                 | Dermato-vénérérologie                                                |
| PU-PH  | <b>LEROUX Dominique</b>                     | Génétique                                                            |
| PU-PH  | <b>LEROY Vincent</b>                        | Gastro-entérologie, hépatologie, addictologie                        |
| PU-PH  | <b>LETOUBLON Christian</b>                  | chirurgie générale                                                   |
| PU-PH  | <b>LEVY Patrick</b>                         | Physiologie                                                          |
| MCU-PH | <b>LONG Jean-Alexandre</b>                  | Urologie                                                             |
| PU-PH  | <b>MACHECOURT Jacques</b>                   | Cardiologie                                                          |
| PU-PH  | <b>MAGNE Jean-Luc</b>                       | Chirurgie vasculaire                                                 |
| MCU-PH | <b>MAIGNAN Maxime</b>                       | Thérapeutique, médecine d'urgence                                    |
| PU-PH  | <b>MAITRE Anne</b>                          | Médecine et santé au travail                                         |
| MCU-PH | <b>MALLARET Marie-Reine</b>                 | Epidémiologie, économie de la santé et prévention                    |
| MCU-PH | <b>MARLU Raphaël</b>                        | Hématologie, transfusion                                             |
| MCU-PH | <b>MAUBON Danièle</b>                       | Parasitologie et mycologie                                           |
| PU-PH  | <b>MAURIN Max</b>                           | Bactériologie - virologie                                            |
| MCU-PH | <b>MCLEER Anne</b>                          | Cytologie et histologie                                              |
| PU-PH  | <b>MERLOZ Philippe</b>                      | Chirurgie orthopédique et traumatologie                              |
| PU-PH  | <b>MORAND Patrice</b>                       | Bactériologie - virologie                                            |
| PU-PH  | <b>MOREAU-GAUDRY Alexandre</b>              | Biostatiques, informatique médicale et technologies de communication |
| PU-PH  | <b>MORO Elena</b>                           | Neurologie                                                           |
| PU-PH  | <b>MORO-SIBILOT Denis</b>                   | Pneumologie                                                          |
| MCU-PH | <b>MOUCHET Patrick</b>                      | Physiologie                                                          |
| PU-PH  | <b>MOUSSEAU Mireille</b>                    | Cancérologie                                                         |
| PU-PH  | <b>MOUTET François</b>                      | Chirurgie plastique, reconstructrice et esthétique, brûologie        |
| MCU-PH | <b>PACLET Marie-Hélène</b>                  | Biochimie et biologie moléculaire                                    |
| PU-PH  | <b>PALOMBI Olivier</b>                      | Anatomie                                                             |
| PU-PH  | <b>PARK Sophie</b>                          | Hémato - transfusion                                                 |
| PU-PH  | <b>PASSAGGIA Jean-Guy</b>                   | Anatomie                                                             |
| PU-PH  | <b>PAYEN DE LA GARANDERIE Jean-François</b> | Anesthésiologie réanimation                                          |
| MCU-PH | <b>PAYSANT François</b>                     | Médecine légale et droit de la santé                                 |

|        |                               |                                                                      |
|--------|-------------------------------|----------------------------------------------------------------------|
| MCU-PH | <b>PELLETIER Laurent</b>      | Biologie cellulaire                                                  |
| PU-PH  | <b>PELLOUX Hervé</b>          | Parasitologie et mycologie                                           |
| PU-PH  | <b>PEPIN Jean-Louis</b>       | Physiologie                                                          |
| PU-PH  | <b>PERENNOU Dominique</b>     | Médecine physique et de réadaptation                                 |
| PU-PH  | <b>PERNOD Gilles</b>          | Médecine vasculaire                                                  |
| PU-PH  | <b>PIOLAT Christian</b>       | Chirurgie infantile                                                  |
| PU-PH  | <b>PISON Christophe</b>       | Pneumologie                                                          |
| PU-PH  | <b>PLANTAZ Dominique</b>      | Pédiatrie                                                            |
| PU-PH  | <b>POLACK Benoît</b>          | Hématologie                                                          |
| PU-PH  | <b>POLOSAN Mircea</b>         | Psychiatrie d'adultes                                                |
| PU-PH  | <b>PONS Jean-Claude</b>       | Gynécologie obstétrique                                              |
| PU-PH  | <b>RAMBEAUD Jacques</b>       | Urologie                                                             |
| MCU-PH | <b>RAY Pierre</b>             | Génétique                                                            |
| PU-PH  | <b>REYT Émile</b>             | Oto-rhino-laryngologie                                               |
| MCU-PH | <b>RIALLE Vincent</b>         | Biostatiques, informatique médicale et technologies de communication |
| PU-PH  | <b>RIGHINI Christian</b>      | Oto-rhino-laryngologie                                               |
| PU-PH  | <b>ROMANET J. Paul</b>        | Ophtalmologie                                                        |
| MCU-PH | <b>ROUSTIT Matthieu</b>       | Pharmacologie fondamentale, pharmaco clinique, addictologie          |
| MCU-PH | <b>ROUX-BUISSON Nathalie</b>  | Biochimie, toxicologie et pharmacologie                              |
| PU-PH  | <b>SARAGAGLIA Dominique</b>   | Chirurgie orthopédique et traumatologie                              |
| MCU-PH | <b>SATRE Véronique</b>        | Génétique                                                            |
| PU-PH  | <b>SAUDOU Frédéric</b>        | Biologie Cellulaire                                                  |
| PU-PH  | <b>SCHMERBER Sébastien</b>    | Oto-rhino-laryngologie                                               |
| PU-PH  | <b>SCHWEBEL-CANALI Carole</b> | Réanimation médicale                                                 |
| PU-PH  | <b>SCOLAN Virginie</b>        | Médecine légale et droit de la santé                                 |
| MCU-PH | <b>SEIGNEURIN Arnaud</b>      | Epidémiologie, économie de la santé et prévention                    |
| PU-PH  | <b>STAHL Jean-Paul</b>        | Maladies infectieuses, maladies tropicales                           |
| PU-PH  | <b>STANKE Françoise</b>       | Pharmacologie fondamentale                                           |
| MCU-PH | <b>STASIA Marie-José</b>      | Biochimie et biologie moléculaire                                    |
| PU-PH  | <b>TAMISIER Renaud</b>        | Physiologie                                                          |
| PU-PH  | <b>TONETTI Jérôme</b>         | Chirurgie orthopédique et traumatologie                              |
| PU-PH  | <b>TOUSSAINT Bertrand</b>     | Biochimie et biologie moléculaire                                    |
| PU-PH  | <b>VANZETTO Gérald</b>        | Cardiologie                                                          |
| PU-PH  | <b>VUILLEZ Jean-Philippe</b>  | Biophysique et médecine nucléaire                                    |
| PU-PH  | <b>WEIL Georges</b>           | Epidémiologie, économie de la santé et prévention                    |
| PU-PH  | <b>ZAOUI Philippe</b>         | Néphrologie                                                          |
| PU-PH  | <b>ZARSKI Jean-Pierre</b>     | Gastro-entérologie, hépatologie, addictologie                        |

## SERMENT D'HIPPOCRATE



*En présence des Maîtres de cette Faculté, de mes chers condisciples et devant l'effigie d'HIPPOCRATE,*

*Je promets et je jure d'être fidèle aux lois de l'honneur et de la probité dans l'exercice de la Médecine.*

*Je donnerai mes soins gratuitement à l'indigent et n'exigerai jamais un salaire au dessus de mon travail. Je ne participerai à aucun partage clandestin d'honoraires.*

*Admis dans l'intimité des maisons, mes yeux n'y verront pas ce qui s'y passe ; ma langue taira les secrets qui me seront confiés et mon état ne servira pas à corrompre les mœurs, ni à favoriser le crime.*

*Je ne permettrai pas que des considérations de religion, de nation, de race, de parti ou de classe sociale viennent s'interposer entre mon devoir et mon patient.*

*Je garderai le respect absolu de la vie humaine.*

*Même sous la menace, je n'admettrai pas de faire usage de mes connaissances médicales contre les lois de l'humanité.*

*Respectueux et reconnaissant envers mes Maîtres, je rendrai à leurs enfants l'instruction que j'ai reçue de leurs pères.*

*Que les hommes m'accordent leur estime si je suis fidèle à mes promesses.*

*Que je sois couvert d'opprobre et méprisé de mes confrères si j'y manque.*

## RÉSUMÉ

**Contexte.** Les microARNs ont émergé comme bio-marqueurs potentiels pour le diagnostic et le pronostic des tumeurs corticosurrénaliennes (TCS). Notre équipe a montré que miR-483-5p et miR-139-5p sont sur-exprimés dans les carcinomes corticosurrénaliens (CCS) (Chabre et al. 2013). Toutefois, le rôle de ces miRNAs dans la pathogenèse des CCS est encore inconnu.

**Objectif.** L'objectif était d'identifier les gènes cibles de miR-483-5p et miR-139-5p dans le CCS et d'étudier leur contribution dans la tumorigenèse corticosurrénalienne.

**Méthodes.** La prédiction des cibles a été réalisée en utilisant les bases de données miRWalk et TargetScan, combinées avec le profil d'expression génique des tumeurs corticosurrénaliennes, publié précédemment (de Reyniès et al. 2009). Le niveau d'expression tumoral de miR-483-5p et miR-139-5p a été mesuré par RT-qPCR dans une cohorte de 28 patients issus de la banque du réseau national COMETE (**C**Ortico- **M**Edullo **T**umeurs **E**ndocrines), et corrélé au niveau d'expression des gènes cibles hypothétiques. La région 3'-non traduite (3'UTR) des gènes cibles a été clonée en aval d'un gène rapporteur Luciferase pour évaluer l'interaction microARN-ARN messager. Le rôle des deux microARNs dans le phénotype malin de la lignée cellulaire NCI- H295R a été étudié par transfection d'inhibiteurs de microARNs.

**Résultats.** Nous avons identifié NDRG2 (N-Myc Downstream-Regulated Gene 2) et NDRG4 comme cibles de miR-483-5p et miR-139-5p, respectivement. L'expression de NDRG2 et NDRG4 dans les CCS était inversement corrélée à l'expression de miR-483-5p et miR-139-5p, respectivement. L'inhibition de miR-483-5p et miR-139-5p dans les cellules NCI-H295R entraîne une augmentation du niveau d'ARNm de NDRG2 et NDRG4. De plus, les transcrits de NDRG2 et NDRG4 sont régulés négativement via un site d'interaction spécifique avec les microARNs. Enfin, l'inhibition de miR-483-5p et de miR-139-5p n'a pas affecté la prolifération ou l'apoptose des cellules NCI- H295R, mais a inhibé leur caractère invasif et leur croissance en gel d'agar sans ancrage cellulaire.

**Conclusion.** Nos données suggèrent que miR-483-5p et miR-139-5p contribuent à l'agressivité des CCS en réprimant l'expression de deux gènes suppresseurs de tumeurs, NDRG2 et NDRG4.

**Mots clefs.** **microARNs, carcinome corticosurrénalien, N-Myc Downstream-Regulated Genes, invasion, miR-483-5p, miR-139-5p**