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Intérêt diagnostique et pronostique de l'immunohistochimie NDRG2 et NDRG4 dans les tumeurs corticosurréaliennes

Bruno Boisson

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

Bruno BOISSON
[Données à caractère personnel]

THÈSE SOUTENUE PUBLIQUEMENT À LA FACULTÉ DE MÉDECINE DE
GRENOBLE*
Le 9 octobre 2017

DEVANT LE JURY COMPOSÉ DE

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Directeur de thèse : Mme le Docteur Nadia CHERRADI

Co-directeur de thèse : Mme le Professeur Nathalie STURM

Membres

M. le Professeur Olivier CHABRE

M. le Professeur Philippe CHAFFANJON

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Madame le Docteur Nadia CHERRADI,

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Sois assurée de ma gratitude et ma considération.

Aux membres du Jury,

Monsieur le Professeur Olivier CHABRE,

Monsieur le Professeur Philippe CHAFFANJON,

Vous me faites l'honneur d'avoir accepté de juger ce travail,

Veillez trouver ici l'expression de mes remerciements les plus sincères.

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État de l'art

Introduction - Épidémiologie

Le carcinome corticosurrénalien (CCS) est une tumeur rare, agressive, dont l'incidence est estimée entre 1 et 2 patients par million d'habitants (soit en France entre 60 et 120 nouveaux cas/an). Le sexe ratio (F/H) varie de 1,5-2,5 :1 (1) avec une distribution bimodale en fonction de l'âge, soit un pic dans la petite enfance et un pic entre 40 et 50 ans (2). Le mauvais pronostic de cette tumeur se traduit par une survie globale de 38,6% à 5 ans après chirurgie, tous stades confondus (3). Dans près de la moitié des cas, ce cancer est diagnostiqué à un stade métastatique avec une survie médiane de moins de 15 mois (3,4). Hormis les prédispositions génétiques, aucun facteur de risque n'a été formellement reconnu, bien que le rôle des œstrogènes ait souvent été suggéré (5,6). Ces prédispositions génétiques sont essentiellement représentées par les mutations germinales TP53, présentes dans 50 à 80% des cas pédiatriques versus 3 à 7% chez l'adulte (7,8). Les syndromes Li-Fraumeni, de Beckwith-Wiedemann et plus rarement de Lynch et la Néoplasie Endocrinienne Multiple de type 1 (NEM 1) sont associés au CCS, sous-tendus par des anomalies génétiques spécifiques (TP 53 ; réarrangements au locus 11p15 ; gènes du Mismatch Repair/MMR ; MENIN respectivement).

Présentation clinique

Un tiers des patients présente un syndrome de masse dû à l'évolution locale de la tumeur tandis qu'un tiers des tumeurs est de découverte fortuite (incidentalome) à la suite d'une imagerie (2,3). Quarante à 60% des patients présentent des signes de sécrétion hormonale (glucocorticoïdes, androgènes, minéralocorticoïdes), parfois difficiles à reconnaître. L'European Network for the Study of Adrenal Tumors (ENSAT) suggère un bilan hormonal complet lors de toute suspicion de CCS, en partie guidé par la clinique, afin de confirmer ou

d'exclure un excès de sécrétions hormonales, de déterminer l'origine corticosurrénalienne de la tumeur, de permettre un suivi hormonal au long cours et d'évaluer la nécessité de la mise en place d'un traitement par hydrocortisone en post-opératoire (tableau 1).

Type d'hormone	Bilan biologique
Glucocorticoïdes (minimum 3 des 4 tests)	<ul style="list-style-type: none"> - Test à la dexaméthasone (1 mg à 23 h), « freinage minute » - Cortisol libre urinaire (urines de 24 h) - Cortisolémie à 8 h - ACTH (plasma)
Stéroïdes sexuels et précurseurs stéroïdiens	<ul style="list-style-type: none"> - DHEA-S sérique - 17-OH-progestérone sérique - Androstènedione sérique - Testostérone sérique - 17β-estradiol sérique (homme et femme ménopausée) - 17 céto- et 17 hydroxy-stéroïdes urinaires (urines de 24 h)
Minéralocorticoïdes	<ul style="list-style-type: none"> - Kaliémie - Ratio aldostérone/rénine (HTA et/ou hypokaliémie)
Catécholamines	<ul style="list-style-type: none"> - Catécholamines urinaires (urines de 24 h) - Méthoxytyramine, méta- et normétanéphrine (plasma)

Tableau 1. Bilan hormonal recommandé par l'ENSAT

Imagerie

L'examen de choix diagnostique et préopératoire est le scanner thoraco-abdominal avec injection de produit de contraste. Il s'attache à déterminer les caractéristiques du CCS : masse surrénalienne hétérogène, volumineuse, mesurant souvent plus de 6 cm (sensibilité, 91%; spécificité, 80%) (9), à contours mal limités, de densité spontanée élevée (> 10 unités Hounsfield) avec prise de contraste intense et persistante après injection de produit de contraste iodé (Wash Out). Enfin, le scanner permet l'évaluation de l'extension locorégionale et est un bon outil de suivi au long cours. Dans les cas douteux au scanner, trois caractéristiques à l'Imagerie par Résonance Magnétique (IRM) évoquent la nature maligne

corticale : un signal T1 iso ou hypointense ; un hypersignal T2 ; une chute de signal hétérogène avec séquence en opposition de phases (10,11). Enfin, la tomographie par émission de positons (TEP)-scan au [¹⁸F] fluorodeoxyglucose (FDG) est complémentaire de l'imagerie conventionnelle, et pratiquement systématiquement réalisée. Le rapport SUVmax (Standard Uptake Value) de la tumeur sur celui du foie permet de distinguer les tumeurs corticosurréaliennes bénignes des CCS avec une sensibilité proche de 100 % et une spécificité de 88% (12). Cependant, le [¹⁸F] FDG PET-scan ne permet pas de distinguer le CCS des métastases, lymphomes ou phéochromocytomes qui possèdent également une hyperactivité métabolique. Il est aussi utile pour le diagnostic des métastases à distance.

Diagnostic anatomopathologique

Bien qu'évoqué en préopératoire devant l'association d'éléments cliniques et radiologiques, le diagnostic définitif est posé par l'analyse anatomopathologique de la pièce opératoire. La ponction biopsie surrénalienne n'est pas utilisée dans la stratégie diagnostique en raison de sa faible valeur diagnostique et du risque de dissémination tumorale liée à la rupture capsulaire.

Le premier temps macroscopique s'attache à la description de la tumeur. Le CCS est d'ordinaire de grande taille (>5cm) et pèse plus de 50g (13). Sa tranche de section focalement jaune-orangé reflète le contenu lipidique des cellules tumorales. Elle est hétérogène, traduisant les remaniements nécrotiques et hémorragiques presque toujours présents. La présence d'une invasion tumorale doit être recherchée à plusieurs niveaux : dans la capsule tumorale, dans la graisse péri-surrénalienne, dans les vaisseaux (veines et lymphatiques).

Les difficultés diagnostiques peuvent être de deux ordres : d'une part établir le diagnostic de malignité d'une tumeur corticosurrénalienne et d'autre part établir l'origine corticosurrénalienne d'une tumeur maligne.

Le score histopronostique de Weiss et ses modifications successives reste à ce jour le système de référence utilisé afin de déterminer le caractère malin ou bénin de la tumeur (14).

Ce score prend en compte 9 critères cytologiques et histologiques : grade nucléaire élevé classé Fürhman 3 ou 4, index mitotique $> 5/50$ champs à fort grossissement (HPF), mitose atypique, nécrose tumorale, architecture diffuse sur plus d'un tiers de la surface tumorale, moins de 25 % de cellules claires sur la surface tumorale, franchissement capsulaire, invasion sinusoïdale et invasion veineuse (1 point par item). Initialement, en 1984, sur une série de 43 tumeurs corticosurréaliennes, un score à 3 classait la tumeur en tumeur de pronostic indéterminé (14). En 1989, sur une cohorte de 42 carcinomes corticosurréaliens, Weiss *et al.* redéfinissent les tumeurs de score 3 comme des tumeurs malignes, sur l'observation d'une récurrence tumorale d'une tumeur de score 3 (15). De plus, la présence de plus de 20 mitoses pour 50 HPF est associé à un pronostic péjoratif (survie globale de 14 mois versus 58 mois), classant ces tumeurs en CCS de haut grade (15). Les deux principales limites du score de WEISS résident dans sa faible reproductibilité inter-observateurs concernant certains critères ($\kappa < 0.6$ pour le grade nucléaire selon Fürhman, l'architecture, l'invasion veineuse et des sinusoïdes) (16) et dans son manque de spécificité puisqu'une tumeur avec un score de 3/9, bien que classée parmi les carcinomes, peut avoir une évolution bénigne. Également, de rares tumeurs avec un score de 2/9 peuvent avoir un comportement malin (17). De ce fait, Aubert *et al.* ont proposé en 2002 un score de Weiss modifié, plus reproductible car réduit à 5 critères (index mitotique, moins de 25 % de cellules claires sur la surface tumorale, mitose atypique, nécrose et invasion capsulaire) et pondéré d'un facteur 2 pour le nombre de mitoses ($> 5/50$ HPF) et la présence de moins de 25 % de cellules claires sur la surface tumorale. Les tumeurs dont le score est égal ou supérieur à 3 sur 7 sont considérées comme malignes (sensibilité 100%, spécificité 96%) (16). Enfin, en 2012, un groupe de pathologistes français s'est attaché à redéfinir précisément les 9 critères du score de Weiss afin d'augmenter la reproductibilité intra et inter-observateur de ce score (18).

Des variantes histologiques des CCS existent et peuvent compliquer la détermination du caractère bénin ou malin. La plus fréquente est la variante oncocytaire définie comme une prolifération de cellules au cytoplasme abondant, granuleux, éosinophile en rapport avec l'accumulation cytoplasmique de mitochondries et de réticulum endoplasmique. Parce que cette variante possède par nature un cytoplasme éosinophile, des atypies nucléaires et une architecture diffuse, le score de Weiss n'est pas adapté car il a tendance à surévaluer son potentiel malin. Des scores spécifiques, tels que celui de Bisceglia, prédisent mieux le potentiel évolutif des tumeurs oncocytaires. Enfin, des variantes myxoïdes ou sarcomatoïdes (19) sont également décrites.

Parmi les autres critères diagnostiques ou pronostiques des CCS, l'index de prolifération cellulaire est le plus reconnu. Il est maintenant bien établi qu'un CCS présente un $Ki67 \geq 5\%$ (20). Beuschlein *et al.* ont montré qu'il s'agissait d'un facteur pronostique indépendant puissant dont la stratification ($Ki67 < 10\%$; $\geq 10\%$ et $< 20\%$; $\geq 20\%$) est associée à un pronostic péjoratif croissant (21).

D'autre part, l'origine corticosurrénaliennne d'une tumeur maligne peut être difficile à établir. Les phéochromocytomes, les métastases de carcinome à cellules claires (rein, foie) ou de mélanome (formes oncocytaires, myxoïdes, sarcomatoïdes) constituent des diagnostics différentiels morphologiques. Le profil immunohistochimique de la tumeur peut alors aider à confirmer sa nature corticosurrénaliennne. Dans ce cadre, l'immunomarquage du steroidogenic factor 1 (SF-1) apparaît comme le marqueur le plus sensible et le plus spécifique (22,23). De plus, la tumeur corticosurrénaliennne n'exprime pas ou focalement les cytokératines et est négative pour la chromogranine A. À contrario, les cellules corticosurrénaliennes expriment la synaptophysine, le CD56, la calrétinine, le melanA et l'inhibine (24,25).

Classification ENSAT

La classification actuelle recommandée pour les CCS est la classification établie par l'European Network for the Study of Adrenal Tumors (ENSAT) en 2008 (26). Elle définit 4 stades : les stades I (≤ 5 cm) et les stades II (≥ 5 cm) sont des tumeurs confinées à la glande surrénalienne. Les stades III sont définis par l'infiltration des tissus adjacents, la présence de métastases ganglionnaires régionales ou de thrombi carcinomateux dans la veine cave ou rénale. Les stades IV regroupent les tumeurs présentant des métastases à distance (tableau 2).

Stage	ENSAT 2008
I	T1, N0, M0
II	T2, N0, M0
III	T1-T2, N1, M0
	T3-T4, N0-N1, M0
IV	T1-T4, N0-N1, M1

ENSAT indicates European Network for the Study of Adrenal Tumors; T1, tumor ≤ 5 cm; T2, tumor > 5 cm; T3, tumor infiltration into surrounding tissue; T4, tumor invasion into adjacent organs or venous tumor thrombus in vena cava or renal vein; N0, no positive lymph nodes; N1, positive lymph node(s); M0, no distant metastases; M1, presence of distant metastasis.

Tableau 2. Classification ENSAT 2008

Cette classification a remplacé la classification UICC (International Union Against Cancer, 2004) car elle permet une meilleure prédiction de la survie et de la récurrence des patients (26). Publiée en 2009 sur la base d'une cohorte allemande de 416 patients suivis sur une moyenne de 36 mois (figure 1) (26), elle a été validée sur une cohorte américaine de 573 patients en 2010 (27).

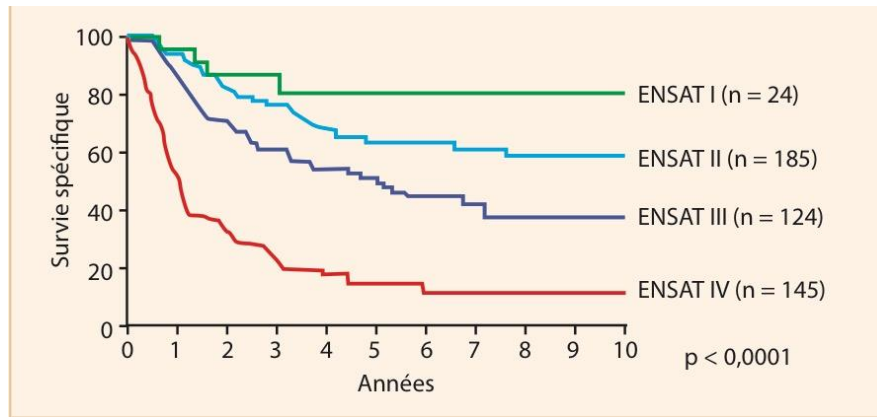


Figure 1. Survie spécifique en fonction du stade tumoral (Classification ENSAT, données issues du German ACC Registry, 2008)

Facteurs pronostiques

Les trois principaux facteurs pronostiques des CCS sont :

- le stade ENSAT avec une survie à 5 ans de 66-82% dans les stades I versus 0-17% dans les stades IV (6,26,27),
- le statut de résection après la chirurgie (R0 ou R1) (28),
- l'intensité de la prolifération cellulaire incluant le compte de mitoses >20 /50 HPF et le Ki67 >10% (21,29).

Traitement

Chirurgie

Le traitement de référence des CCS localisés (stades ENSAT I et II) repose en première intention sur une exérèse chirurgicale large dont le but est l'obtention d'une résection monobloc complète R0 (Annexe 1). Les stades ENSAT III pour lesquels un tel objectif est envisageable restent éligibles à ce traitement (30). Il a été démontré qu'une intervention dans un centre de référence avec une large expérience opératoire (>10 surrénalectomies pour cancer par an) améliore d'un facteur 2 la survie sans récurrence et la survie globale (31). La chirurgie par laparotomie est recommandée, mais la résection par laparoscopie, bien que sujette à controverse, est possible sous certaines conditions, en centre spécialisé (stade localisé, taille < 8cm, avec résection de la graisse péri-surrénale et sans rupture capsulaire, absence de signe d'infiltration à l'imagerie préopératoire) (30). Un curage ganglionnaire locorégional systématique améliore le staging et la survie (32). La chirurgie itérative semble le meilleur traitement des récurrences locales, en cas de récurrence tardive (>12 mois) et de chirurgie initiale R0 (33). Dans les situations d'emblée métastatiques, l'option chirurgicale reste envisageable, à évaluer au cas par cas, en fonction de différents paramètres

(masse tumorale, nombre d'organes métastatiques, rapidité d'évolution, hypersécrétion hormonale non contrôlée par un traitement médicamenteux).

Traitement adjuvant

Le taux élevé de récurrence après chirurgie (de 19 à 34% selon les séries) justifie l'emploi d'un traitement adjuvant, médical ou la radiothérapie (20).

Mitotane

Le mitotane est le traitement médical de référence des CCS. Son utilisation en situation adjuvante a en effet démontré une amélioration en terme de survie sans récurrence chez les patients de stade I-II R0 opérés versus absence de traitement adjuvant (34). Une étude prospective randomisée multicentrique internationale (ADIUVO) est en cours pour évaluer de manière prospective l'efficacité de ce traitement adjuvant versus surveillance seule, chez les patients avec risque faible ou intermédiaire de progression tumorale (Stade I-II, Ki67 \leq 10%) après chirurgie (35). Le mitotane est également indiqué dans les stades localement avancés (stade III) dont l'exérèse chirurgicale complète n'est pas envisageable, ainsi que dans les stades métastatiques (stade IV).

Chimiothérapie

En cas de tumeur rapidement évolutive, il est recommandé en première ligne d'utiliser une association Etoposide, Doxorubicine, Cisplatine (EDP) et Mitotane (30).

Radiothérapie

La radiothérapie du lit tumoral est envisagée chez les patients avec une résection chirurgicale incomplète R1, bien que n'ayant jamais prouvé son efficacité sur la survie globale (36).

Suivi

Actuellement, le suivi ne fait pas l'objet de recommandations internationales. Cependant, un suivi initial régulier tous les 3 mois incluant examen clinique, bilan hormonal, scanner thoraco-abdominal est préconisé (30).

Pathologie moléculaire

Voies de signalisation

Les deux altérations les plus fréquentes retrouvées dans le CCS sont d'une part la surexpression du facteur insulin-like growth factor 2 (IGF2) dans environ 90% des cas, liée à un réarrangement spécifique du locus du gène IGF2 dans la région 11p15, avec perte de l'allèle maternel et duplication de l'allèle paternel (37), et d'autre part, dans un tiers des cas, l'activation constitutive de la voie de signalisation Wnt/ β -caténine, le plus souvent par mutation somatique activatrice du gène de la β -caténine (CTNNB1) (38). Enfin, des mutations somatiques inactivatrices de TP53 sont présentes dans 50 à 80% des cas pédiatriques versus 3 à 7% chez l'adulte (7,8). Plus récemment, des études pangénomiques se sont intéressées à l'analyse des anomalies chromosomiques, du transcriptome (expression des gènes), du méthylome (anomalies de méthylation de l'ADN) et du miRNome (microARNs) (39,40).

Anomalies chromosomiques

Les altérations chromosomiques du CCS, étudiées notamment par hybridation génomique comparative et SNP-array (single nucleotide polymorphism), ont retrouvé de nombreux gains, impliquant les chromosomes 5, 7, 12 et 19 mais également des pertes, en particulier au niveau du chromosome 22. L'évaluation de ces altérations peut fournir des informations concernant le pronostic, certaines combinaisons d'événements étant associées à des pronostics différents au sein des CCS (39,41).

Transcriptome

L'étude du transcriptome permet de distinguer les CCS des adénomes, notamment via l'étude des niveaux d'expression de gènes aléatoires lors de l'analyse non supervisée de clusters de gènes (37,42) ou lors de l'étude de deux clusters de gènes, le cluster "IGF2" (huit gènes) et le cluster "Stéroïdogénèse" (14 gènes) (43). De plus, des études ont montré l'émergence de deux sous-groupes différents en terme de survie globale au sein des CCS, que ce soit par l'analyse non supervisée du transcriptome (cluster C1A de pronostic plus péjoratif que le cluster C1B) ou lors de l'étude du niveau d'expression de deux gènes BUB1B et PINK 1 (44).

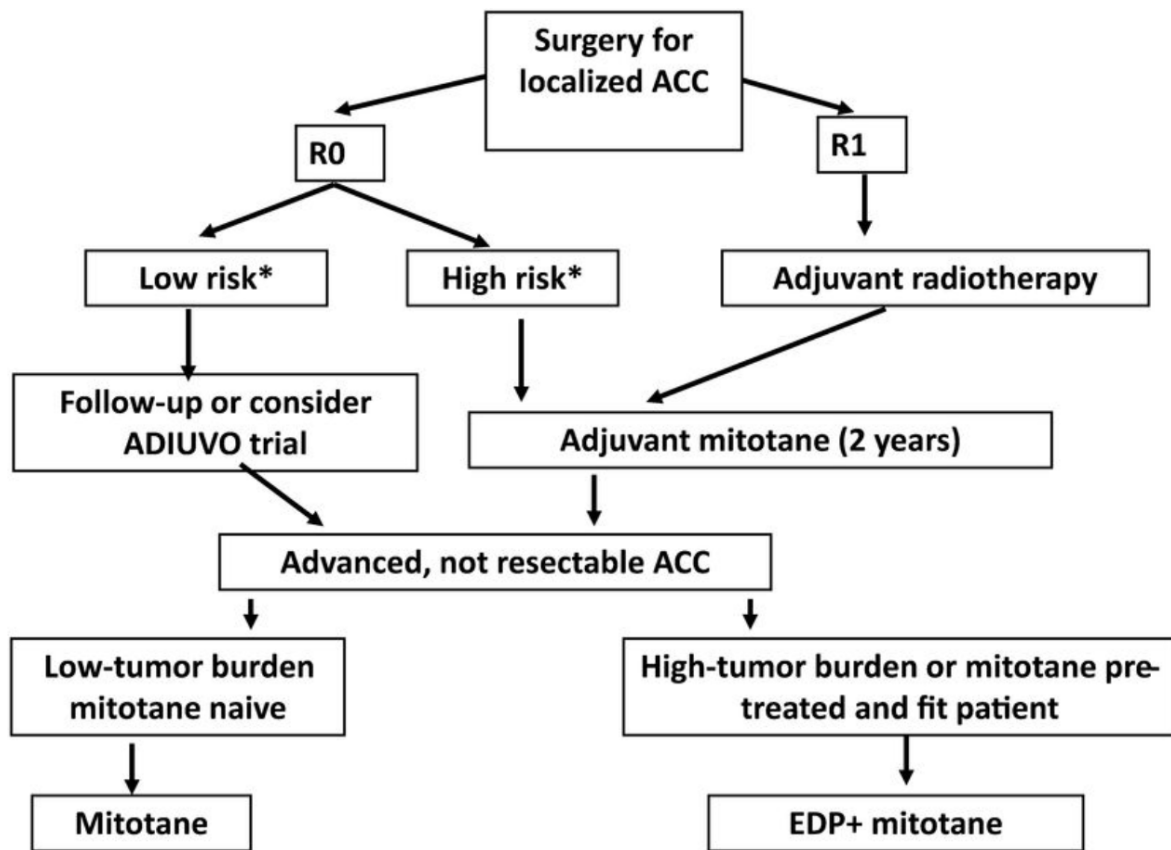
Méthylome

Les analyses de méthylation ont été initialement décrites dans le cancer du côlon avec l'identification de tumeurs hyperméthylées, appelé CIMP (CpG island methylator phenotype) (45). Depuis, ce phénotype CIMP a été décrit dans de nombreux autres types tumoraux (vessie, sein, rein, ovaires...) et notamment dans les CCS, globalement hyperméthylés par rapport aux adénomes. L'hyperméthylation présente dans les CIMP est responsable de la sous-expression d'un grand nombre de gènes. La classification hiérarchique non supervisée sépare également les CCS en 2 sous-groupes, différents par leur niveau de méthylation (CIMP-high, CIMP-low), l'hyperméthylation étant associée à un mauvais pronostic (46).

MiRNome

Les microARNs (miARNs), découverts en 1993 chez le nématode *C. elegans*, sont des petits ARNs non codants double-brin de 21 à 24 nucléotides. Ils jouent un rôle fondamental dans la régulation post-transcriptionnelle de l'expression des gènes. Environ 60% des gènes codants pour des protéines seraient régulés par des miARNs. Ces derniers agissent en s'appariant à une séquence complémentaire de l'ARN messager du gène cible au niveau de la région 3'-non traduite (3'UTR) et entraînent une répression de l'expression des gènes par dégradation de

l'ARNm cible ou inhibition de la traduction (47). Plus rarement, une activation transcriptionnelle et/ou traductionnelle a été observée. Les miARNs régulent l'expression de gènes impliqués dans des processus physiologiques tels que la prolifération cellulaire, la différenciation, l'apoptose, la migration et l'invasion cellulaire. Actuellement, près de 2000 miARNs ont été identifiés (48). Plusieurs études se sont intéressées au profil d'expression des miARNs dans les tumeurs corticosurréaliennes (49–53). Elles ont révélé des différences d'expression entre adénomes et CCS, notamment une hyperexpression quasi-constante de miR-483-5p dans le CCS. De manière intéressante, le gène codant pour ce miARN se situe dans l'intron 2 du gène *IGF2*, le niveau d'expression de miR-483-5p étant corrélé à celui d'*IGF2* (49). MiR-139-5p, localisé en 11q13.4 dans le second intron du gène de la phosphodiesterase 2A, est également hyperexprimé dans les CCS de mauvais pronostic (39,51). Récemment, deux gènes cibles de miR-483-5p et miR-139-5p ont été identifiés comme étant *NDRG2* (N-Myc Downstream-Regulated Gene 2) situé en 14q11.2 et *NDRG4*, situé en 16q21- q22.1, respectivement (54). Cette famille de gènes joue un rôle important dans la biologie du cancer, notamment comme suppresseur de tumeurs et intervient dans le cycle cellulaire (55). A l'inverse, certains miARNs sont sous-exprimés dans les CCS, tels que miR-195 et miR-335. De la même manière que pour le transcriptome, l'analyse non supervisée du miRNome a montré son efficacité quant à la distinction entre adénome et CCS, et les différents niveaux d'expression de certains miARNs identifient des sous-types de CCS de pronostic variable (39,49,50,52). Enfin, l'étude des miARNs circulants miR-483-5p et miR-195 a montré une association entre leur profil d'expression sérique et la survie globale ou la survie sans récurrence, conférant à la recherche de ces miARNs dans le sérum un caractère pronostique très intéressant (51).



*Low-risk ACC: stage I–II and Ki67 expression in $\leq 10\%$ of neoplastic cells.

*High-risk ACC: stage III or Ki67 expression in $> 10\%$ of neoplastic cells.

Annexe 1. Recommandations pour le traitement des carcinomes corticosurréaliens.

Figure extraite de *Adrenal Cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up* – Ann Oncol. 2012

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ORIGINAL ARTICLE

Value of NDRG2 and NDRG4 as immunohistochemical markers for the diagnosis and prognosis of adrenocortical carcinoma

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List of abbreviations

ACA: AdrenoCortical Adenoma

aACC: aggressive AdrenoCortical Carcinoma

naACC: non-aggressive AdrenoCortical Carcinoma

IHC: ImmunoHistoChemistry

ISH: *In Situ* Hybridization

miRNA: microRNA

NDRG: N-Myc Downstream-Regulated Genes

RT-qPCR: quantitative Real Time PCR

Abstract

AdrenoCortical Carcinoma (ACC) is a rare and aggressive cancer with poor prognosis and few therapeutic options. Diagnosis of malignancy can be challenging for pathologists and prognostic of ACC remains difficult to predict. MiR-483-5p and miR-139-5p were shown to be upregulated in ACC compared with adrenocortical adenoma (ACA), and N-myc Downstream-Regulated Gene 2 (NDRG2) and NDRG4 were identified as targets of miR-483-5p and miR-139-5p, respectively. In this study, we investigated NDRG2 and NDRG4 expression using immunohistochemistry (IHC), as well as miR-483-5p and miR-139-5p expression using *in situ* hybridization (ISH), by a H-score-like method on formalin-fixed and paraffin-embedded adrenocortical tissue sections. These semi-quantitative approaches were correlated with mRNA and miR expression measured by Real Time quantitative PCR (RT-qPCR) and protein levels determined by western blot in frozen tissues. Ten ACA and twenty ACC were retrospectively selected. ACC were divided into two groups, one consisting of aggressive ACC (aACC) as opposed to non-aggressive ACC (naACC). NDRG2 displayed a significant lower expression in ACC compared with ACA ($p < 0.001$) and a score of 152.5 was determined as the cutoff value which best discriminated ACA from ACC with a sensitivity of 100% and a specificity of 95%. NDRG4 expression under 35 allowed to discriminate between aACC and naACC with a sensitivity of 100% and a specificity of 75%. A strong correlation was observed between the scores determined by IHC and their expression measured by RT-qPCR and protein levels determined by western blot. Moreover, NDRG2 expression measured by IHC was inversely correlated with its regulatory miRNA expression detected by ISH. In conclusion, our study demonstrates that NDRG2 and NDRG4 are potential useful immunomarkers for ACC diagnosis and prognosis that could be applied as a routine complementary histopathological tool.

Keywords: adrenocortical carcinoma, diagnosis, immunohistochemistry, microRNAs, miR-483-5p, N-myc Downstream-Regulated Genes, prognosis, RT-qPCR

Introduction

AdrenoCortical Carcinoma (ACC) is a rare and aggressive cancer with poor prognosis and few therapeutic options. Adrenocortical tumors have a prevalence of at least 3% in a population over the age of 50 years (1,2) whereas ACC is less frequent with an estimated incidence of 1 to 2 per million people and a 5 years-survival rate of approximately 40% (3–6). There is a bimodal distribution depending on the age, a first peak appearing during childhood and a second peak during the fourth and fifth decades (7).

The Weiss scoring system is the most commonly used method to assess malignancy of adrenocortical tumors (8). Most recently, several studies have demonstrated the value of Ki67 expression in differentiating benign from malignant tumors (9,10) and as a predictive factor of recurrence (11). However, some overlaps between benign and malignant tumors have been described (12,13), with few reported Weiss 2 tumors having a metastatic evolution (14).

The overall survival of patients with ACC is variable. Patients with ACC with limited extension to the gland, classified stages I and II in the European Network for the Study of Adrenal Tumors (ENSAT), have a much better prognosis than those classified stages III and IV, with locally extended tumors or metastases. Furthermore, prognosis of patients having a metastatic evolution at onset is also variable, as for those staged ENSAT stage I or II having a recurrence after surgery (15). These different evolutive profiles support the hypothesis of different underlying onco-biologic mechanisms.

Through the past 15 years, extensive studies of the molecular characteristics of ACC have led to a better comprehension of the biology of adrenocortical tumors. Pan-genomic analyses allowed significant progress in the characterization of the methylome, the transcriptome and the miRNome of ACC. Apart from IGF2 overexpression, TP53 tumor suppressor gene inactivation and mutations of the Wnt/ β -Catenin signaling pathway (16–18), other molecular features have also been associated with malignancy of adrenocortical tumors

(19,20). Based on the study of the transcriptome, two subgroups of ACC have been identified among the malignant group C1. Tumors of group C1B had a better prognosis compared to group C1A. This C1B group also had a malignant profile compared to the benign C2 group (recurrence 15% versus 2%) with a superior Weiss score (21).

Current data underline the advantages of using microRNAs (miRNAs) to establish the malignant profile and the subgroup classification among ACC. MiRNAs are small non-coding RNA of twenty nucleotides that play a key role in the regulation of gene expression at the post-transcriptional level through their binding to the 3'-untranslated region (UTR) of the target mRNA. Located within intron 2 of *IGF2* gene, miR-483-5p is almost constantly overexpressed in ACC (22–29) and correlated with IGF2 expression (25). Overexpression of miR-139-5p was also found in aggressive ACC (aACC ; recurrence or death \leq 3 years or metastatic tumor) but not in non-aggressive ACC (naACC ; no recurrence or recurrence $>$ 3 years) (29). Interest in miRNAs also relied upon their stability over mRNAs, allowing their study in the serum. Interestingly, miR-483-5p was shown to be dramatically increased in the serum of patients with ACCs and has been suggested as a potential noninvasive biomarker for aggressiveness in ACC (29).

We have previously shown that miR-483-5p and miR-139-5p target genes which belong to the N-myc Downstream Regulated Gene family (NDRG) (30). This family counts 4 genes located on distinct chromosomes which are involved in cellular proliferation and differentiation. MiR-483-5p and miR-139-5p interact with the 3'-untranslated region of NDRG2 and NDRG4, respectively (30). Using Real Time quantitative PCR (RT-qPCR), Agosta *et al.* showed in a cohort of 10 adrenocortical adenomas (ACA) and 18 ACC that NDRG2 expression was significantly lower in ACC compared to ACA. On the other hand, NDRG4 under-expression was able to discriminate between aggressive and non-aggressive ACC. Very interestingly, NDRG2 and NDRG4 re-expression or miR-483-5p and miR-139-

5p inhibition impaired adrenocortical cancer cell migration and invasion (30). These findings are consistent with other data regarding NDRG2 expression in many other cancers (mammary carcinoma, colorectal, gastric, glioblastoma,...) (31–34). By contrast, data concerning NDRG4 remain scarce and it seems to have a tissue-dependent oncogenic function.

In this study, we investigated for the first time NDRG2 and NDRG4 expression in Formalin-Fixed and Paraffin-Embedded (FFPE) adrenocortical tumor tissue samples using immunohistochemistry (IHC), as well as miR-483-5p and miR-139-5p expression using *in situ* hybridization (ISH), in correlation with their mRNA and miR expression measured by RT-qPCR and protein levels determined by western blot, in frozen tissues.

Methods

Patients and tumor samples

Paired FFPE and frozen adrenocortical tumor tissue samples were retrospectively obtained from a cohort of 30 patients who underwent adrenalectomy at Centre Hospitalier Universitaire Grenoble Alpes (CHUGA) from 1994 to 2014. Twenty malignant and ten benign tumors were selected. These samples belong to the tumor bank of the department of Pathology (DACP collection declared to Ministry of Higher Education and Research DC-2008-584 Collection 1), affiliated to the Biological Resources Center (CRB) of the CHUGA. Informed signed consents for the analysis of the tumor tissue and access to the data were obtained from all the patients operated after the 10th August 2007 (Decree No 2007-1220 on sampling, storage and preparation of components of the human body for scientific purposes and amending the Public Health Code).

All 30 tumors had full clinical and pathological data. Adrenocortical tumor were scored from 0 to 9, according to the Weiss system (Weiss 1984) by a unique expert pathologist (N.S). Tumors with a Weiss score of 0–2 were considered benign, whereas tumors with a Weiss score of 3–9 were considered malignant (35). Staging was established according to the ENSAT staging system (Fassnacht, *et al.* 2009).

Adrenocortical carcinoma (ACC) samples were divided into two groups: a first group consisting of aggressive tumors (aACC, recurring tumors or metastatic tumors at diagnosis, n=12) and a second group consisting of non-aggressive tumors (naACC, non-recurring tumors, n=8). Tumors that did not recur within the first 3 years after surgery were considered as non-recurring carcinoma.

Immunohistochemistry

Immunohistochemistry staining was performed on 3 µm FFPE tissue sections using a Benchmark XT autostainer (Roche/Ventana Medical Systems, Tucson AZ, USA). After deparaffinization, antigen retrieval was carried out with Cell Conditioning 1 (CC1) standard antigen retrieval buffer (Tris/Borate/EDTA pH 8.4) for 1h at 98°C. After 1h incubation with primary antibody, revelation was achieved by using UltraView DAB detection kit (Ventana Medical Systems).

Primary antibodies against NDRG2 (Cell Signaling Technology, #5667), NDRG4 (Clone D4A6, Cell Signaling Technology, #9039) and Ki67 (Clone MIB 1, Agilent, M7240) were used at optimized concentrations of 1.4 µg/ml, 1.6 µg/ml and 0.9 µg/ml, respectively. Adjacent normal adrenocortical tissue served as a positive control for NDRG2 and NDRG4 expression. Negative controls were performed using ChromPure Rabbit IgG whole molecule (1.4 µg/ml, Jackson Immunoresearch).

We scored NDRG2 and NDRG4 staining using a semi-quantitative H-score-like method that combines the percentage of stained cells with the intensity of staining (36–38). The score was given as the sum of the percent staining multiplied by an ordinal value corresponding to the intensity level (0 = none, 1 = weak, 2 = moderate, and 3 = strong), or $[1 \times (\% \text{ cells } 1+) + 2 \times (\% \text{ cells } 2+) + 3 \times (\% \text{ cells } 3+)]$. The resulting score ranged from 0 to 300, with 0 representing complete absence of staining, while a score of 300 corresponded to diffuse strong staining of tumor cells.

Ki67 proliferative index was calculated on 1000 tumor cells in areas of hot spots, using image capture and manual counting on NIS-Elements imaging software version 3.10.

MicroRNA *in situ* hybridization

miRCURY LNA detection probes for human miR-483-5p (5'-TCCCTTCTTTCCTCCCGTCTT-3'), U6-positive control and scrambled negative control, which were all 5'-digoxigenin- and 3'-digoxigenin-labelled, were purchased from Exiqon (Vedbaek, Denmark). MicroRNA *in situ* hybridization was performed on 6- μ m FFPE sections using the miRCURY LNATM microRNA ISH Optimization Kit (EXIQON, #90009) according to manufacturer's instructions (instruction manual v3.0).

Briefly, tissue sections were deparaffinized, treated with 15 μ g/ml proteinase K for 30min at 37°C and washed twice with PBS. After prehybridization at 59°C for 1 h, hybridization was performed at 59°C for 21 h. Slides were washed in decreasing SSC buffer concentrations (SSC buffer 20X concentrated, Sigma, S6639) at 59°C and then in 0,2X SSC at room temperature. After blocking (2% sheep serum and 1% bovine serum albumin in PBS containing 0,1% Tween-20) at room temperature for 1 h, sections were incubated for 1h at room temperature with sheep anti-digoxigenin-alkaline phosphatase (Roche, Cat. no. 11 093274910) diluted 1:800 in antibody diluent solution (1% sheep serum and 1% bovine serum albumin in PBS containing 0,05% Tween-20). Slides were washed three times in PBS 0,1% Tween, and then incubated 2h in dark at 30°C with alkaline phosphatase substrate (NBT-BCIP ready to use tablet (Roche, Cat. n°11697471001) diluted in milli-Q water with 0,2 mM levamisol (Fluka, cat. N°31742)). Reaction was stopped by incubating slides with KTBT buffer (50mM Tris, 150mM NaCl, 10mM KCl). Slides were counterstained with Nuclear Fast Red, dehydrated and mounted.

Based on our experience in immunohistochemical scoring, we adopted a slightly similar method to score *in situ* hybridization. Intensity levels were defined as follow: 0 = absence of staining, 1 = weak staining often covering the nucleus, 2 = moderate staining predominant in the cytoplasm and 3 = strong cytoplasmic staining (Supplementary Figure

S1). The score was given as the sum of the percent staining multiplied by an ordinal value corresponding to the intensity level, or $[1 \times (\% \text{ cells } 1+) + 2 \times (\% \text{ cells } 2+) + 3 \times (\% \text{ cells } 3+)]$

RNA extraction and RT-qPCR

Total RNA was isolated from frozen tumor tissue using mirVANA PARIS Kit (Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer's instructions. MiRNA levels in cell lysates were measured by RT-qPCR using TaqMan miRNA assays (Applied Biosystems, Thermo Fisher Scientific). Ten nanograms of cell total RNA were reverse-transcribed using the TaqMan miRNA Reverse Transcription kit and miRNA-specific stem-loop primers (Applied Biosystems, Thermo Fisher Scientific) in a 15 μ l-RT reaction (composed of 0.15 μ l 100 mM dNTPs mix, 1.5 μ l 10x RT buffer, 0.19 μ l RNase inhibitor (20 units/ μ l), 4.16 μ l H₂O, 1 μ l multiscribe reverse transcriptase, and 5 μ l 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 TaqMan 2X Universal PCR Master Mix and the appropriate TaqMan MicroRNA Assay Mix for each miRNA of interest (Assay ID: hsa-miR-483-5p: 002338; hsa-miR-139-5p: 002289; RNU48: 001006). Briefly, 4.5 μ l of 2.5-fold diluted RT product was combined with 5.5 μ l of PCR assay reagents to generate a PCR volume of 10 μ l. 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 in duplicate. The RNU48 RNA was used as an endogenous control for normalization. Normalized expression was calculated using the comparative Ct method and fold changes were derived from the $2^{-\Delta\Delta C_t}$ values for each miRNA.

N-myc Downstream-Regulated Gene 2 and 4 (NDRG2 and NDRG4) expression was assessed with 1 µg of total RNA extract from tissue samples 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 H₂O, 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. 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. Expression of NDRG2 and NDRG4 was normalized to RPL13A and HPRT as housekeeping genes. Normalized expression was calculated as described above. Correlation between miRNA 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. Only 25 frozen samples of our initial cohort were available.

Western blot analysis

Proteins were extracted from frozen tissue samples in automated beads-containing homogenization tubes and Cell Disruption Buffer (Applied Biosystems, Thermo Fisher Scientific). They were quantitated with microBCA protein Assay kit (Thermo Fisher Scientific). 15 µg of total protein per sample were SDS-PAGE-resolved and electrophoretically transferred onto a polyvinylidene fluoride (PVDF) membrane using the Trans-Blot Turbo™ transfer system (Bio-Rad) according to the manufacturer's instructions. 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-Tubulin (1:40000, kindly provided by Dr A. Andrieux (Grenoble Institute of Neuroscience, Grenoble, France), rabbit anti-NDRG2 or 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 Scientific) or HRP-conjugated goat anti-rabbit IgG (1:3000, Thermo Fisher Scientific). The PVDF sheet was washed as above and the antigen-antibody complex revealed by Enhanced Chemiluminescence using the detection kit and the ChemiDoc MP imaging system from Bio-Rad. Only 25 frozen samples of our initial cohort were available.

Statistical analyses

Analyses were performed using SPSS software package, version 23.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism software version 7.01 (San Diego, CA, USA). Comparison between groups was performed with the non-parametrical Mann–Whitney U or Kruskal–Wallis tests for quantitative variables and with the Fischer’s exact test for qualitative variables. Correlation between quantitative variables was assessed by the Spearman coefficient. Overall survival (OS) was defined as time elapsed between surgery of the primary tumor and death or last follow-up visit. Recurrence-free survival (RFS) was defined as time elapsed between surgery and the first evidence of recurrence or last follow-up without evidence of disease. Survival curves were obtained with Kaplan–Meier estimates and compared with the log-rank test. Cox proportional hazards regression was used to determine multivariate hazard ratios (HRs) for selected potential predictors of RFS and OS. Factors with $p < 0.05$ in univariate analysis for RFS and OS were selected and included in the multivariate regression analysis using Cox proportional hazards regression model, which was performed to achieve adjusted hazard ratio (HR) to determine prognostic factors of RFS and OS. A $p < 0.05$ was considered significant for all analyses.

Results

Patient cohort and characteristics

The main clinical and pathological characteristics of the 20 patients with ACC and the 10 patients with ACA are summarized in Table 1 and Table 2, respectively. There was no statistical difference in sex and age between the patients with ACC and those with ACA (median age of 44 and 42 years, respectively). However, ACC were significantly larger and heavier than ACA (9.5 vs 4 cm; 160.5 vs 20.5 g, respectively; both $p < 0.001$). Moreover, median Ki67 was statistically higher in ACC than ACA (13.1 vs 1.2%, $p < 0.001$). Mitotic index more than 5 per 50 high-power fields (HPF) was observed in 16 of 20 ACC and none of the 10 ACA ($p < 0.001$) (Table 3).

Among ACC, 8 were non-aggressive and 12 were aggressive, according to the definition of aggressiveness. Although age and sex were not different between these 2 groups, tumor size and weight, Weiss score, mitotic index, Ki67 proliferative index and ENSAT stage were statistically higher in aACC than in naACC (Table 4). In particular, all of the aACC had a Weiss score superior to 6 and were classified as III or IV ENSAT stages. The Ki67 proliferative index was lower in the naACC group (5.2% vs 17.2%, $p < 0.001$) and only one naACC had a mitotic index higher than 20 per 50 HPF. Among the aACC group, the median follow-up was 11 months (range 0.9-80.5 months) and lower than in the naACC group (88.6 months, range 42.8-150 months, $p < 0.001$). One patient was lost to follow up after the primary surgery (patient 11) and patient 15, who had the larger and heavier tumor of the cohort, died from postoperative complications. Half of the aACC patients were non-metastatic at the time of diagnosis ($n=6$) but all of them had recurrence and only one patient was still alive 5 years after primary surgery. By contrast, only two naACC patients had a recurrence at 39.8 months and 54 months, and 87.5% were still alive 5 years after primary surgery.

Assessment and diagnostic value of NDRG2 expression by immunohistochemistry

Immunohistochemical study revealed robust NDRG2 expression in ACA and adjacent normal cortex (Figure 1D). An ACA specimen treated with nonspecific rabbit IgG were completely nonreactive. By contrast, ACC featured weak heterogeneous or complete loss of NDRG2 expression as compared to ACA and adjacent tissue. (Figures 1E and 1F). These findings demonstrate that NDRG2 protein is differentially expressed between ACC and ACA or normal adrenal cortex.

We next compared NDRG2 score between adenomas, carcinomas (combined aACC and naACC), naACC and aACC separately. Results of scoring are summarized Table 5. NDRG2 displayed a significant lower expression in ACC compared with ACA ($p < 0.001$) and its expression was lower in naACC compared with ACA ($p = 0.02$, Figure 2A). However, NDRG2 expression was not statistically different between aACC and naACC.

We next determined the diagnostic value of NDRG2. ROC analyses indicated high diagnostic accuracy for NDRG2 in discriminating ACA from ACC (area under the curve (AUC) of 0.973 for NDRG2, 95% CI: 0.916-1, $p < 0.001$) and high diagnostic accuracy for NDRG2 in discriminating between ACA and naACC (AUC of 0.931, 95% CI: 0.974-1, $p = 0.002$) (Figures 3A and 3B, respectively). For a convenient use of NDRG2 immunohistochemical scoring in clinical practice, based on ROC curves, we determined the optimal cutoff value that should be used for separating these groups. The optimal cutoff value for NDRG2 was 152.5, in discriminating both ACA from ACC (sensitivity: 100%, specificity: 95%) and ACA from naACC (sensitivity: 100%, specificity: 87.5%) (Figure 3B).

These data showed NDRG2 as a reliable immunohistochemical marker to distinguish ACA from ACC.

Assessment and diagnostic value of NDRG4 expression by immunohistochemistry

Immunohistochemistry showed a robust expression of NDRG4 in ACA and adjacent normal cortex (Figure 1G). By contrast, aACC featured complete loss of NDRG4 expression compared to ACA and adjacent tissue (Figure 1I), whereas naACC demonstrated a weak heterogeneous staining, slightly different from the absence of expression in aACC (Figure 1H).

NDRG4 displayed a significant lower expression in ACC compared with ACA ($p=0.018$). Remarkably, NDRG4 expression showed a significant lower expression in aACC compared with naACC ($p=0.043$) while it was not different between naACC and ACA (Figure 2B).

ROC analyses indicated high diagnostic accuracy for NDRG4 in discriminating ACA from ACC (AUC of 0.835, 95% CI: 0.687-0.983, $p=0.003$) and high diagnostic accuracy for NDRG4 in discriminating between naACC and aACC subgroups (AUC of 0.885, 95% CI: 0.735-1, $p=0.004$). The optimal cutoff value for NDRG4 in discriminating ACA from ACC was 140 (sensitivity: 100%, specificity: 75%) (Figure 3C). To distinguish naACC from aACC, 35 was the optimal cutoff value for NDRG4 with a sensitivity of 100% and a specificity of 75% (Figure 3D).

These results pointed NDRG4 as a reliable immunohistochemical marker to distinguish aACC from naACC.

Assessment and diagnostic value of miR-483-5p expression by *in situ* hybridization

In situ hybridization study revealed moderate to strong miR-483-5p expression in ACC, represented by a blue-purple cytoplasmic staining (Figures 4C and 4D). By contrast, adenoma showed a weak miR-483-5p expression, which was slightly similar to the expression in normal adrenal cortex (Figures 4A and 4B).

We found a significant overexpression of miR-483-5p in ACC and aACC compared with ACA ($p=0.035$ and $p=0.012$, respectively) (Figure 2C).

ROC analyses indicated high diagnostic accuracy for miR-483-5p in discriminating ACA from ACC (AUC of 0.810, 95% CI: 0.646-0.974, $p=0.006$) and the optimal cutoff value for miR-483-5p in discriminating ACA from ACC was 170 (sensitivity: 75%, specificity: 90%) (Figure 3E).

These data demonstrated that miR-483-5p expression by *in situ* hybridization could help in distinguishing ACA from ACC.

Assessment of miR-139-5p expression by *in situ* hybridization

Unfortunately, despite several attempts, we were not able to implement the technique and obtain a proper or informative *in situ* hybridization signal for miR-139-5p. Tests are still ongoing.

Correlation between NDRG2, NDRG4 and miR-483-5p expression determined by RT-qPCR analysis and their respective scores in immunohistochemistry

In agreements with previous results obtained in an independent cohort (Agosta et al, submitted), NDRG2 transcripts were down-regulated in ACC as compared with ACA ($p=0.004$) and NDRG4 transcripts were down-regulated in aACC as compared with naACC ($p=0.046$, Figure 5) A strong positive correlation was found between NDRG2 expression

determined by RT-qPCR and NDRG2 score determined by immunohistochemistry in adrenocortical tumors ($r= 0.684$, $p<0.001$, Figure 6A). Similar results were obtained for NDRG4 expression determined by RT-qPCR and NDRG4 score determined by immunohistochemistry ($r= 0.795$, $p<0.001$, Figure 6B). A moderate positive correlation was found between miR-483-5p expression determined by RT-qPCR and miR-483-5p score determined by in situ hybridization, ($r= 0.460$, $p= 0.021$, Figure 6C).

Correlation between NDRG2 and NDRG4 protein levels determined by western blot analysis and their respective scores in immunohistochemistry

Protein levels of NDRG2 and NDRG4 were also determined by western blot on frozen tissue samples, and the results were consistent with that of immunochemistry and RT-qPCR analyses. Both protein levels of NDRG2 and NDRG4 were found decreased in ACC compared with ACA ($p=0.011$ and $p=0.005$, respectively, Figure 7), and protein level of NDRG4 was decreased in aACC compared with naACC ($p=0.017$). A strong positive correlation was found between NDRG2 and NDRG4 expression levels determined by western blot and their respective scores determined by immunohistochemistry, in adrenocortical tumors ($r= 0.752$, $p<0.001$; $r= 0.811$, $p<0.001$, respectively; not shown).

Relationship between miR-483-5p and NDRG2 in adrenocortical tumors

Because NDRG2 was identified as target of miR-483-5p (30), we evaluated the relationship between their scores determined by semi-quantitative method. We found a significant negative correlation between their expressions in adrenocortical tumors ($r= -0.53$, $p= 0.003$, Figure 8).

Relationships between NDRG4 and NDRG2 expression and clinicopathological features of ACC

We next examined the associations of NDRG4 and NDRG2 expression in ACC with the clinicopathological characteristics (Table 6). The previously determined cutoff value of NDRG4 score was used to define a high expression (>35) or low expression (≤ 35), and the median of NDRG2 score served to define low (≤ 10) and high expression (>10). Low NDRG4 expression was remarkably associated with high Weiss score ($p=0.014$), high mitotic index ($p=0.045$), high proliferating index assessed by Ki67 ($p=0.013$) and recurrence or metastasis positive status ($p=0.011$). Low NDRG2 expression was associated with gender ($p=0.005$), tumor size ($p=0.032$), tumor weight ($p=0.005$) and ENSAT stage ($p=0.026$).

Moreover, high NDRG4 score was inversely correlated with Ki67 ($r=-0.534$, $p=0.015$, Figure 9A) and Weiss score ($r=-0.596$, $p=0.006$, Figure 9B). Similarly, high NDRG2 score was negatively correlated with tumor size ($r=-0.521$, $p=0.019$, Figure.9C) or tumor weight ($r=-0.666$, $p=0.001$, Figure 9D). As expected, no correlation was found between NDRG4 expression and tumor size or weight, or between NDRG2 expression and Ki67 or Weiss score.

Recurrence and survival analyses

Although our cohort was relatively small, we attempted to analyze RFS and OS as a function of NDRG4 and NDRG2 expression levels. The patients with tumors that were already metastatic before surgery were excluded from the recurrence-free survival analyses. Univariate analysis for all relevant clinical and histopathological parameters were performed (Table 7). Parameters associated with high risk of recurrence included tumor weight ≥ 160 g ($p=0.004$), Ki67 $>10\%$ and mitotic index $\geq 20/50$ HPF (both $p<0.001$), ENSAT stage III and low NDRG4 score ($p=0.023$ and $p<0.001$, respectively). Tumor size and weight, Ki67, mitotic

index, ENSAT stage and low NDRG4 score were significantly associated with poor overall survival. In particular, Kaplan-Meier analysis, according to ROC-derived cutoff value of NDRG4, revealed that the group with low NDRG4 score had a significant higher risk of recurrence and worse overall survival (HR: 8.173, 95% CI: 8.403-2285, Log-rank test $p < 0.001$; HR: 5.937, 95% CI: 1.571-22.43, Log-rank test $p < 0.001$) (Figure 10). NDRG4 score and ENSAT stage III were then combined, as well as NDRG4 score and Ki67>10%. As shown Figure 11, both overall survival and recurrence-free survival were significantly reduced in ENSAT stage III patients with low NDRG4 score compared with ENSAT stage III patients with high NDRG4 score (median overall survival 11 versus 71 months respectively, HR: 4.077, 95% CI: 0.513-32.39, Log-rank test $p = 0.022$; median recurrence-free survival 2.7 versus 46.9 months respectively, HR: 5.13, 95% CI :0.557-47.27, Log-rank test $p = 0.01$). Similarly, patients with Ki67>10% and low NDRG4 expression had also a poorer survival compared with those with a Ki67>10% and high NDRG4 expression (median overall survival 10.65 versus 57.55 months respectively, HR: 2.676, 95% CI: 0.853-8.40, Log-rank test $p = 0.043$). By contrast, there was no significant difference in terms of recurrence-free survival for patients with Ki67>10% with low NDRG4 score compared with those with high NDRG4 score ($p = 0.11$).

All variables associated with patient recurrence or survival at a $p < 0.05$ level of significance were included in multivariate Cox regression analysis (Table 8). None of the parameters were significantly and independently associated with an increased risk of recurrence or death, but low NDRG4 score tended to be an unfavorable prognostic factor in OS ($p = 0.064$).

Discussion

Diagnosis of localized ACC is still a complex issue and remains challenging, especially with increased detection of adrenocortical tumors, often found incidentally during workup for unrelated indication (2,39). Although several different scoring systems have been proposed to assess malignancy in adrenocortical tumors, Weiss score remains the most commonly used in separating benign from malignant adrenocortical neoplasms (8). However, low Weiss scores of 2 and 3 generate ambiguous results, especially in small localized tumors with Weiss score of 3 and in large tumors with Weiss score of 2 without invasive features. Indeed, several studies already reported that ACC with Weiss score of 3 often do not recur, or described adrenocortical tumors with Weiss score of 2 with lung metastases (14,40). This grey zone needs further investigation to obtain a better characterization. Among immunohistochemical markers, Ki67 expression has been indicated as the most useful marker of malignancy for adrenocortical tumors (9,41). However, some overlaps between benign and malignant lesions have also been described (12,13).

We report here the first study demonstrating the potential diagnostic and prognostic values of NDRG2 and NDRG4 immunohistochemical expression in adrenocortical tumors, which could be easily used in routine clinical practice on FFPE tissue and which is well correlated with gene expression measured by RT-qPCR and protein levels determined by western blot.

The current study has investigated a cohort of patients with ACC treated at a single institution, with a median age of 44 years, a sex ration (F/M) of 1.9/1, 30% of metastasis at diagnosis and 5-year survival rate of 42%. These features were similar to those reported in the literature (5,6,42,43).

We scored NDRG2, NDRG4 and miR-483-5p using a semi-quantitative H-score-like method, which allowed to establish a score ranges from 0 to 300 reflecting heterogeneous

protein expression. This scoring system revealed underexpression of NDRG2 and NDRG4 and overexpression of miR-483-5p in ACC compared with ACA and adjacent normal cortical tissue, consistent with previous results obtained from RT-qPCR analysis on frozen samples.

Indeed, many authors previously reported that a panel of miRNAs is altered in ACC (25,44,45). Among dysregulated miRNAs, miR-483-5p and miR-139-5p were found highly upregulated in ACC, and might contribute to ACC aggressiveness (46). Using quantitative RT-PCR, Agosta *et al.* demonstrated that miR-483-5p and miR-139-5p target NDRG2 and NDRG4 transcripts, respectively, both significantly under-expressed in ACC as compared to ACA (30,46). Human NDRG2 and NDRG4 are located on chromosomes 14q11.2 and 16q21–q22.1, respectively. Multiple studies have shown a tumor suppressor role for NDRG2 in cancer (32–34,47). NDRG2 down-regulation have been described in a variety of human cancer cell lines including breast, colon, and lung cancer cells (48,49). Much less is known concerning NDRG4 expression and function in cancer, but it seems to have tumor-suppressive or oncogenic functions depending on the tissue type (50–53). NDRG4 methylation at the promoter CpG islands in colorectal cancer cell lines and primary colorectal cancer tissue was associated with its diminished mRNA and protein expression (50).

As mentioned above, previous determinations of NDRG2 and NDRG4 expression levels were conducted using mRNA quantification measured by RT-qPCR (Agosta *et al.*, 2017, submitted). Importantly, we demonstrate here a strong correlation between expression levels determined by RT-qPCR analysis of our cohort and our scores determined on paraffin-embedded tissues by a semi-quantitative H-score–like method. These significant correlations indicate a strong biological link between mRNA and protein expression and suggest that the semi-quantitative H-score–like method is an efficient approach to evaluate immunohistochemical signals. Moreover, we also showed that our results obtained by IHC were the same as those observed by western blot analysis, with a strong correlation between

these two methods, thus supporting the idea that we have efficiently evaluated immunohistochemical signals. Both RT-qPCR assays and ISH demonstrated upregulation of miR-483-5p in ACC compared to ACA but the correlation between RT-qPCR data and ISH scores was moderate. This finding may reflect a more difficult assessment of hybridization signals on paraffin-embedded tissues. Indeed, although miRNAs seem to be more stable and less prone to degradation than mRNAs, we were not able to detect miR-139-5p using ISH. All conditions tested on FFPE tissues resulted in a negative staining. Considering that only one study reported miR-483-3p (the complementary strand of miR-483-5p) ISH in ACC (28), we hypothesize that this methodology is far from being validated. At this point, we are still improving our protocol.

Moreover, we found a negative correlation between miR-483-5p and NDRG2 expression levels, in agreement with our previous study reporting NDRG2 as targets of miR-483-5p, with low NDRG2 expression inversely correlated with high expression of miR-483-5P in ACC (30).

Our study showed that NDRG2 immunohistochemistry was the best marker to discriminate between benign tumors and malignant ones, with high sensitivity (100%) and specificity (95%), using the cut-off value of 152.5. Tumors with some suspicious features but with a low Weiss score could benefit from this immunomarker assessment.

We identified NDRG4 by IHC as a new potent prognostic marker that accurately discriminates between non-recurring and recurring/metastatic ACC, with sensitivity of 100% and specificity of 75%, using the cut-off value of 35. These findings are in agreement with Chabre *et al.* (29) who previously demonstrated a significant overexpression of its regulatory miR, miR-139-5p, in aACC compared with naACC.

Our clinicopathological study revealed that low NDRG4 expression remarkably associated with high proliferation index and recurrence or metastasis status, suggesting a role in proliferation or invasion. Interestingly, Melotte *et al.* observed that colorectal carcinoma cell lines overexpressing NDRG4 displayed a reduction in proliferation and invasion, whereas no effect was observed on migration (50). The down-regulation of NDRG4 in tumor endothelial cells also suggests the involvement of this family member in angiogenesis (54).

Our study revealed that Ki67, mitotic index and ENSAT stage were associated with high risk of recurrence and poor overall survival, in agreement with numerous previous reports (15,55–58). Soon *et al.* (2009b) and Chabre *et al.* (2013) reported that some miRNAs in ACC tumor tissues or in serum were associated with survival. In our cohort, we showed that NDRG4, a target of miR-139-5p, is a prognostic factor of which low expression is associated with an increased risk of recurrence and a worse overall survival. Moreover, patients who had an ENSAT stage III or a Ki67 superior to 10 percent and a low NDRG4 expression in immunohistochemistry displayed worst overall survival than those with high NDRG4 expression. We also observed that within ENSAT stage III tumors, low NDRG4 expression was associated with shorter recurrence-free survival. Although these findings were observed on the same cohort used to determine NDRG4 cutoff value, they suggest that NDRG4 immunohistochemistry could be used as a valuable prognostic factor. Further studies on an independent cohort are needed to confirm the prognostic value of NDRG4.

Although our cohort was small, we attempted to perform a multivariate analysis. Though it tended to be significant, we did not identify NDRG4 as an independent prognostic factor in multivariate analysis as also observed for mitotic index, Ki67 and ENSAT stage. However, these latter are well-established prognostic factors (15,55–58). We hypothesize that a likely reason for our finding is the small size of the cohort which results in a lack of

statistical power. Further studies with larger sample sizes are needed to establish the role of NDRG4 as an independent prognostic factor in multivariate analysis.

In conclusion, our study demonstrates that NDRG2 and NDRG4 detected by immunohistochemistry are potential useful markers for diagnosis and prognosis of ACC that could be used as a routine complementary histopathological tool. Our data suggest that NDRG2 is a strong marker for diagnosis of malignancy and that NDRG4 is a promising marker to predict prognosis of adrenocortical carcinoma.

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Tables and figures legends

Table 1. Clinicopathological characteristics of the patients with adrenocortical carcinoma

Table 2. Clinicopathological characteristics of the patients with adrenocortical adenoma

Table 3. Comparison of the main characteristics (age, sex, tumor size and weight, mitotic index, and Ki67) of the patients with ACC with those of the patients with ACA.

Table 4. Clinicopathological data of the 2 groups of patients with ACC, dichotomized into aggressive and non-aggressive carcinomas.

Table 5 MiR-483-5p, NDRG4 and NDRG2 expression as determined by in situ hybridization and immunohistochemistry, respectively.

Table 6. Correlations between clinicopathological features and expression of NDRG4 and NDRG2 in adrenocortical carcinomas.

Table 7. Univariate analysis for RFS and OS in the ACC cohort.

Table 8. Multivariate analysis (cox regression) for RFS and OS in the ACC cohort.

Figure 1. Immunohistochemical expression of NDRG2 and NDRG4 in adrenocortical tumors. HES stained sections of adrenocortical tumors are represented in the left column, with normal adrenal cortex (N) and tumoral zone (T) (A, B, C). NDRG2 expression was retained in normal adrenal cortex and in ACA (D) while it was lost or diminished in both naACC (E) and aACC (F). NDRG4 expression was heterogeneous in ACA (G) and naACC (H) while it was strongly diminished in aACC (I).

Figure 2. NDRG2 (A), NDRG4 (B) and miR-483-5p (C) expressions in adrenocortical tumors by immunochemistry and *in situ* hybridization. Statistical analysis was performed using Kruskal-Wallis test for unpaired data and $p < 0,05$ was considered significant (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

Figure 3. Diagnostic values of NDRG2, NDRG4 and miR-483-5p scores. ROC curves analyses of NDRG2, NDRG4 and miR-483-5p expression for discrimination between ACA

and ACC (A, C and E respectively). (B) ROC curves analyses of NDRG2 expression for discrimination between naACC and ACA. (D) ROC curves analyses of NDRG4 expression for discrimination between naACC and aACC.

Figure 4. MiR-483-5p in situ hybridization in adenoma (A, B) and carcinoma (C, D). Adenoma (ACA) showed weak miR-483-5p expression, slightly similar to normal adrenal cortex (N), whereas carcinoma (ACC) demonstrated a strong cytoplasmic staining compared to normal adrenal cortex. Magnification x4 (A, C) and x10 (B, D).

Figure 5. NDRG2 (A) and NDRG4 (B) expressions in adrenocortical tumors in RT-qPCR. Statistical analysis was performed using Kruskal-Wallis test for unpaired data and $p < 0,05$ was considered significant (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

Figure 6. Correlation between NDRG2 (A) and NDRG4 (B) expression determined by quantitative RT-PCR analysis (RT-qPCR) and their scores determined by IHC, in adrenocortical tumors (ACA and ACC). (C) Correlation between miR-483-5p expression determined by RT-qPCR analysis and miR-483-5p score determined by ISH, in adrenocortical tumors.

Figure 7. Protein levels of NDRG2 (A, C) and NDRG4 (B, D) determined by western blot analysis in adrenocortical tumors. β -Actine was used as a loading control. Statistical analysis was performed using Kruskal-Wallis test for unpaired data and $p < 0,05$ was considered significant (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

Figure 8. Correlation between miR-483-5p expression determined by *in situ* hybridization and NDRG2 expression determined by immunohistochemistry, in adrenocortical tumors (ACA and ACC).

Figure 9. Correlations between NDRG4 and Ki67 (A) or Weiss score (B) and between NDRG2 and tumor size (C) or tumor weight (D) were analyzed using Spearman's test, in ACC.

Figure 10. Kaplan-Meier analyses according to NDRG4 expression levels in ACC. (A) Recurrence-free survival as a function of NDRG4 levels. (B) Overall survival as a function of NDRG4 levels.

Figure 11. Prognostic values of ENSAT stage III combined with NDRG4 score, and Ki67>10% combined with NDRG4 score, in ACC. Kaplan-Meier estimates are presented for (A) overall survival and (B) recurrence-free survival for ENSAT stage III and (C) overall survival and (D) recurrence-free survival for Ki67>10%.

Supplementary Figure S1. Representative intensity levels for miR-483-5p detected by in situ hybridization. 0: complete absence of cytoplasmic or nuclear staining (A, B). 1: Weak staining often covering the nuclei (C, D). 2: moderate staining predominant in the cytoplasm (E, F). 3: Strong cytoplasmic staining (G, H). Magnification x4 (A, C, E) and x10 (B, D, F).

Patient ID	Age	Sex	Tumor size (mm)	Tumor weight (g)	Weiss score	ENSAT stage	ACC subgroup	Mitotic index (/50 HPF)	Ki67 (%)	Recurrence (Y/N)	RFS (months)	Metastasis at diagnosis	Status	Survival (months)	Follow-up (years)
1	25	M	70	137	3	III	na	≤5	5.3	N	-	N	Alive	-	12.5
2	71	F	90	267	5	III	na	≤5	3.1	Y	39.8	N	Dead	42.9	3.6
3	16	F	65	90	4	II	na	≤5	3.7	N	-	N	Alive	-	7.1
4	28	F	30	16	7	I	na	≤5	6.3	N	-	N	Alive	-	9.7
5	63	F	50	55	8	III	na	>5 <20	6.9	N	-	N	Alive	-	8.9
6	67	M	110	145	5	III	na	≥20	16.4	Y	54.0	N	Dead	71.1	5.9
7	42	F	55	60	5	II	na	>5 <20	3.8	N	-	N	Alive	-	7.7
8	23	F	40	9	3	I	na	>5 <20	5.1	N	-	N	Alive	-	5.3
9	61	M	105	286	7	IV	a	≥20	10.2	-	-	Y	Dead	19.1	1.6
10	41	M	120	500	9	III	a	≥20	16.3	Y	2.7	N	Dead	3.2	0.3
11	88	F	110	60	9	IV	a	>5 <20	16.7	-	-	Y	LTFU	-	-
12	64	F	55	46	8	IV	a	≥20	10.3	-	-	Y	Dead	10.3	0.9
13	46	M	100	176	8	IV	a	≥20	20.3	-	-	Y	Dead	23.4	1.9
14	31	F	140	305	7	III	a	≥20	10.4	Y	20.0	N	Dead	80.5	6.7
15	46	F	150	1060	9	III	a	≥20	17.6	-	-	N	Dead	0.9	0.1
16	38	F	100	240	8	III	a	≥20	19.7	Y	8.3	N	Dead	44.1	3.7
17	65	F	100	250	9	IV	a	≥20	45.5	-	-	Y	Dead	2.0	0.2
18	59	M	120	290	9	IV	a	≥20	15.8	-	-	Y	Dead	1.9	0.2
19	2	M	90	210	9	III	a	≥20	18	Y	14.9	N	Dead	17.9	1.5
20	34	F	90	115	7	III	a	≥20	26	Y	1.3	N	Dead	11.0	0.9

F, female; M, male; na, non-aggressive ACC group; a, aggressive ACC group; HPF, high-power field; RFS, recurrence-free survival; N, no; Y, yes; LTFU, lost to follow-up.

Table 1. Clinicopathological characteristics of the patients with adrenocortical carcinoma

Patient ID	Age	Sex	Tumor size (mm)	Tumor weight (g)	Weiss score	Mitotic index (/50 HPF)	Ki67 (%)	Follow-up (mo)	Status
21	36	F	40	15	1	≤5	1.5	95.9	Alive
22	42	F	20	10	0	≤5	1	51.4	Alive
23	32	F	25	8	0	≤5	1.2	25.8	Alive
24	51	F	29	8	1	≤5	1.6	43.2	Alive
25	37	M	40	26	2	≤5	0.9	3.6	Alive
26	52	F	60	74	0	≤5	1.2	1.7	Alive
27	62	M	50	34	0	≤5	0.7	1.1	Alive
28	74	F	53	15	0	≤5	0.7	1.5	Alive
29	42	F	20	28	0	≤5	1.8	19.8	Alive
30	33	F	60	36	2	≤5	1.8	14.3	Alive

Table 2. Clinicopathological characteristics of the patients with adrenocortical adenoma

	Malignant (n= 20)	Benign (n=10)	p value
Median age, y (range)	44 (2-88)	42 (32-74)	0,94
Sex (F/M)	13/7	8/2	0,675
Median tumor size, cm (range)	9.5 (3,0-15,0)	4 (2,0-6,0)	< 0,001
Median tumor weight, g (range)	160.5 (9-1060)	20.5 (8-74)	< 0,001
Mitotic index > 5	16	0	< 0,001
Median Ki67, % (range)	13,1 (3,1-45,5)	1,2 (0,7-1,8)	< 0,001

p-values in bold are considered significant.

Table 3. Comparison of the main characteristics (age, sex, tumor size and weight, mitotic index, and Ki67) of the patients with ACC with those of the patients with ACA.

	aACC (n= 12)	naACC (n=8)	p value
Median age, y (range)	46 (2-88)	35 (16-71)	0,56
Sex (F/M)	7/5	6/2	0,642
Median tumor size, cm (range)	10,3 (5,5-15)	6 (3-11)	0,004
Median tumor weight, g (range)	245 (46-1060)	75 (9-267)	0,017
Weiss score ≤6 >6	0 12 (100%)	6 (75%) 2 (25%)	< 0,001
Mitotic index <5 ≥5 <20 ≥20	0 1 (8,3%) 11 (91,7%)	4 (50%) 3 (37,5%) 1 (12,5%)	0,0015
Median Ki67, % (range)	17.2 (10,20-45,50)	5.2 (3,10-16,40)	< 0,001
ENSAT stage stage I or II stage III or IV	0 12 (100%)	4 (50%) 4 (50%)	0,014
Metastasis at diagnosis	6 (50%)	0	
Median follow-up ^a , mo (range)	11 (0,9-80,50)	88.6 (42,80-150)	< 0,001
Recurrence ^b	5 (100%)	2 (25%)	0,02
5-year recurrence free survival ^b	0 (0%)	6 (75%)	< 0,01
5-year survival rate ^a	1 (9%)	7 (87,5%)	< 0,01

^a One patient lost to follow-up in aACC group; ^b Excluding patients with metastasis at diagnosis and a patient who died of postoperative complications.

Mo, months; aACC, aggressive adrenocortical carcinoma; naACC, non-aggressive adrenocortical carcinoma.

p-values in bold are considered significant.

Table 4. Clinicopathological data of the 2 groups of patients with ACC, dichotomized into aggressive and non-aggressive carcinomas.

Patient ID	Age	Sex	Tumor subgroup	Weiss score	Ki67 (%)	MiR-483-5p score	NDRG4 score	NDRG2 score
1	25	M	naACC	3	5,3	230	40	0
2	71	F	naACC	5	3,1	270	180	0
3	16	F	naACC	4	3,7	70	90	60
4	28	F	naACC	7	6,3	200	260	150
5	63	F	naACC	8	6,9	150	70	0
6	67	M	naACC	5	16,4	110	40	10
7	42	F	naACC	5	3,8	180	250	95
8	23	F	naACC	3	5,1	110	215	220
9	61	M	aACC	7	10,2	210	0	0
10	41	M	aACC	9	16,3	180	0	0
11	88	F	aACC	9	16,7	280	20	20
12	64	F	aACC	8	10,3	190	25	30
13	46	M	aACC	8	20,3	190	25	10
14	31	F	aACC	7	10,4	240	110	0
15	46	F	aACC	9	17,6	200	40	0
16	38	F	aACC	8	19,7	230	245	60
17	65	F	aACC	9	45,5	190	15	20
18	59	M	aACC	9	15,8	100	30	10
19	2	M	aACC	9	18	260	0	0
20	34	F	aACC	7	26	210	30	20
21	36	F	ACA	1	1,5	110	210	230
22	42	F	ACA	0	1	140	175	200
23	32	F	ACA	0	1,2	160	235	230
24	51	F	ACA	1	1,6	60	275	220
25	37	M	ACA	2	0,9	220	170	220
26	52	F	ACA	0	1,2	90	270	155
27	62	M	ACA	0	0,7	40	210	200
28	74	F	ACA	0	0,7	110	190	210
29	42	F	ACA	0	1,8	120	210	220
30	33	F	ACA	2	1,8	140	200	260

F, female ; M, male ; naACC, non-aggressive adrenocortical carcinoma ; aACC, aggressive adrenocortical carcinoma ; ACA, adrenocortical adenoma.

Table 5. MiR-483-5p, NDRG4 and NDRG2 expression as determined by *in situ* hybridization and immunohistochemistry, respectively.

Groups	NDRG4			NDRG2		
	Low expression	High expression	p-value	Low expression	High expression	p-value
Gender			0.160			0.005
Male	5	2		7	0	
Female	4	9		4	9	
Age, years						
Median	59	38	0.304	46	38	0.642
Tumor size, cm						
Median	100	70	0.221	105	65	0.032
Tumor weight, g						
Median	210	137	0.423	267	60	0.005
Weiss score			0.014			>0.999
≤6	0	6		3	3	
>6	9	5		8	6	
Mitotic index			0.045			0.34
<5	0	4		2	2	
≥5 <20	1	3		1	3	
≥20	8	4		8	4	
Ki67, %			0.013			>0.999
Median	16.7	6.3		15.8	10.3	
ENSAT stage			0.094			0.026
stage I-II	0	4		0	4	
Stage III-IV	9	7		11	5	
Recurrence or metastasis			0.011			0.35
Yes	9	4		8	5	
No	0	6		2	4	

p-values in bold are considered significant.

Table 6. Correlations between clinicopathological features and expression of NDRG4 and NDRG2 in adrenocortical carcinomas.

	RFS ^a			OS		
	HR	95% CI	p value	HR	95% CI	p value
Tumor size ≥ 80 mm	1.725	0.259-11.48	0.508	13.26	4.463-39.37	0.005
Tumor weight ≥ 160 g	7.157	1.319-38.84	0.004	5.626	1.827-17.32	0.002
Ki67, >10%	14.67	2.868-75.03	<0.001	15.11	5.058-45.14	<0.001
Mitotic index, ≥20	14.67	2.868-75.03	<0.001	15.11	5.058-45.14	<0.001
ENSAT stage I-II vs III	5.881	1.278-27.07	0.023			
I-II vs III-IV				4.675	1.435-15.23	0.011
I-II-III vs IV				4.104	0.824-20.43	0.005
NDRG4 score ≤ 35	8.173	8.403-2285	<0.001	5.937	1.571-22.43	<0.001
NDRG2 ≤10 (median)	2.165	0.490-9.563	0.341	1.875	0.533	0.286

^a Except patients with metastasis at diagnosis

RFS, recurrence-free survival; OS, overall survival; HR, hazard ratio; 95% CI, 95% confidence interval.

p-values in bold are considered significant.

Table 7. Univariate analysis for RFS and OS in ACC cohort.

	RFS^a			OS		
	HR	95% CI	p value	HR	95% CI	p value
Tumor size ≥ 80 mm				2.261	0.070-73.135	0.646
Tumor weight ≥ 160 g	5.018	0.330-76.379	0.246	1.22	0.225-6.615	0.818
Ki67, >10%	6.536	0.527-81.139	0.144	2.03	0.085-48.511	0.662
Mitotic index, ≥20	6.536	0.527-81.139	0.144	2.03	0.085-48.511	0.662
ENSAT stage I-II vs III	1760708	0	0.986			
I-II vs III-IV				3981954	0	0.989
I-II-III vs IV				0.849	0.166-4.355	0.845
NDRG4 score ≤ 35	4.847	0.480-48.977	0.181	10.3	0.869-122.094	0.064

^a Except patients with metastasis at diagnosis

RFS, recurrence-free survival; OS, overall survival; HR, hazard ratio; 95% CI, 95% confidence interval.

Table 8. Multivariate analysis (cox regression) for RFS and OS in ACC cohort.

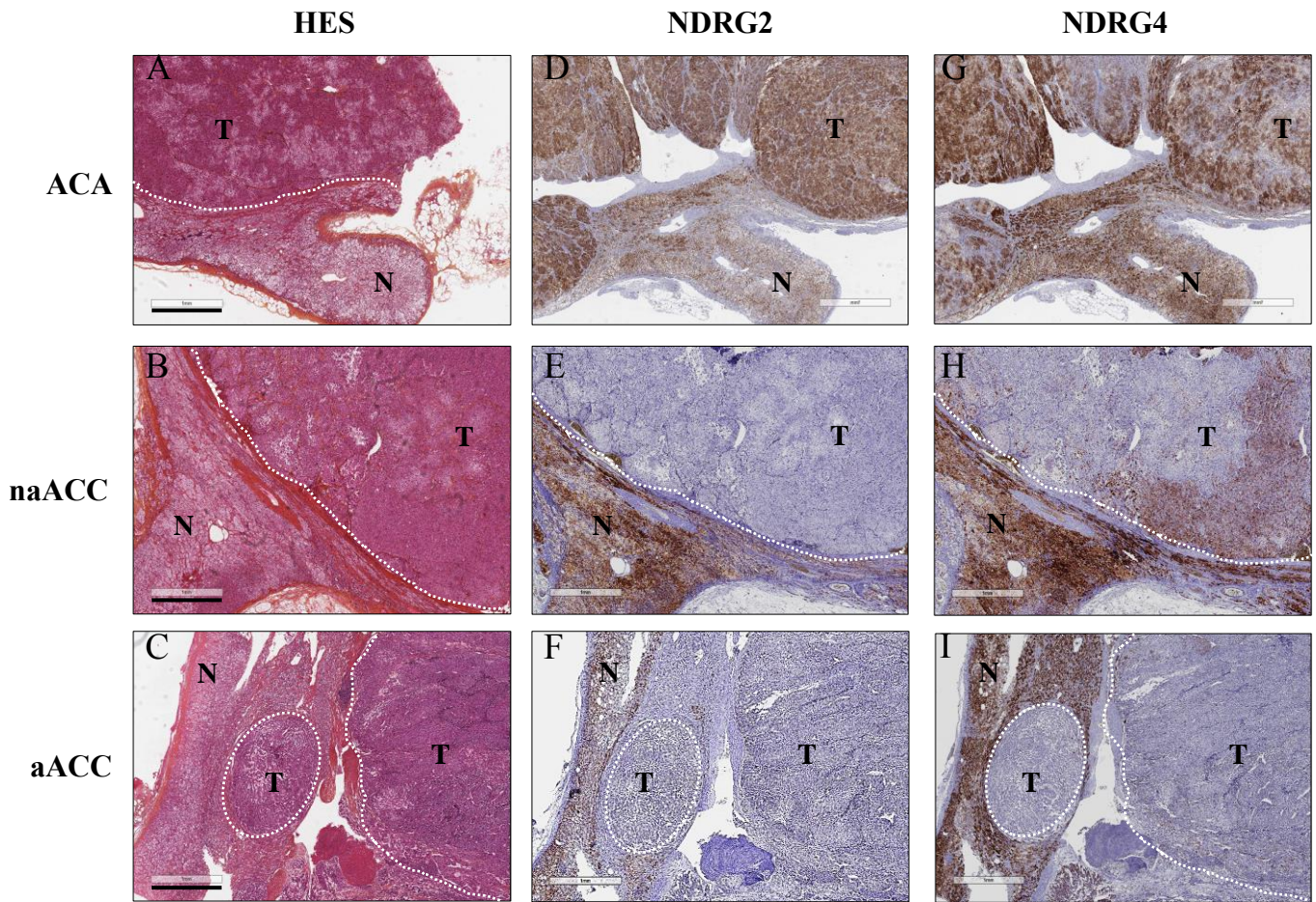


Figure 1. Immunohistochemical expression of NDRG2 and NDRG4 in adrenocortical tumors. HES stained sections of adrenocortical tumors are represented in the left column, with normal adrenal cortex (N) and tumoral zone (T) (A, B, C). NDRG2 expression was retained in normal adrenal cortex and in ACA (D) while it was lost or diminished in both naACC (E) and aACC (F). NDRG4 expression was heterogeneous in ACA (G) and naACC (H) while it was strongly diminished in aACC (I).

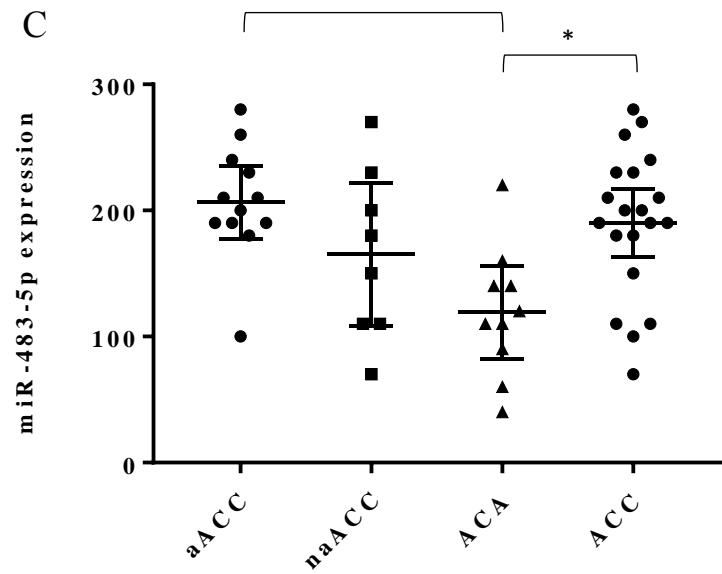
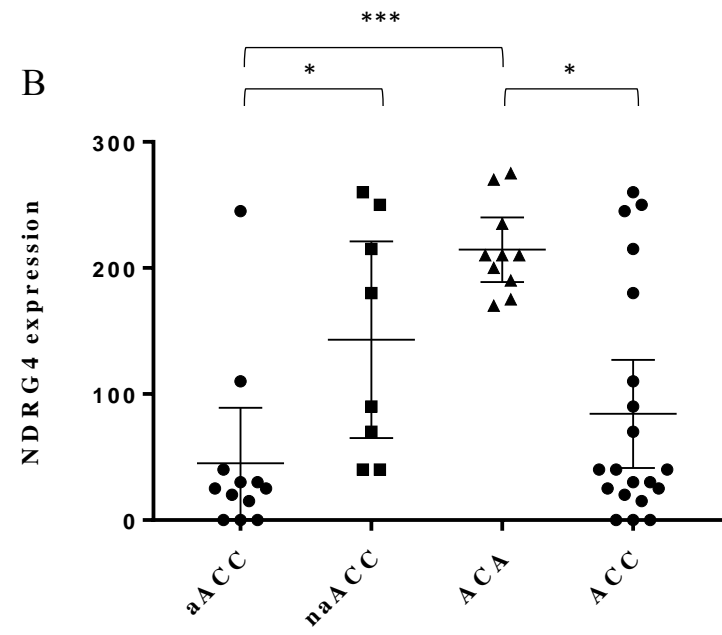
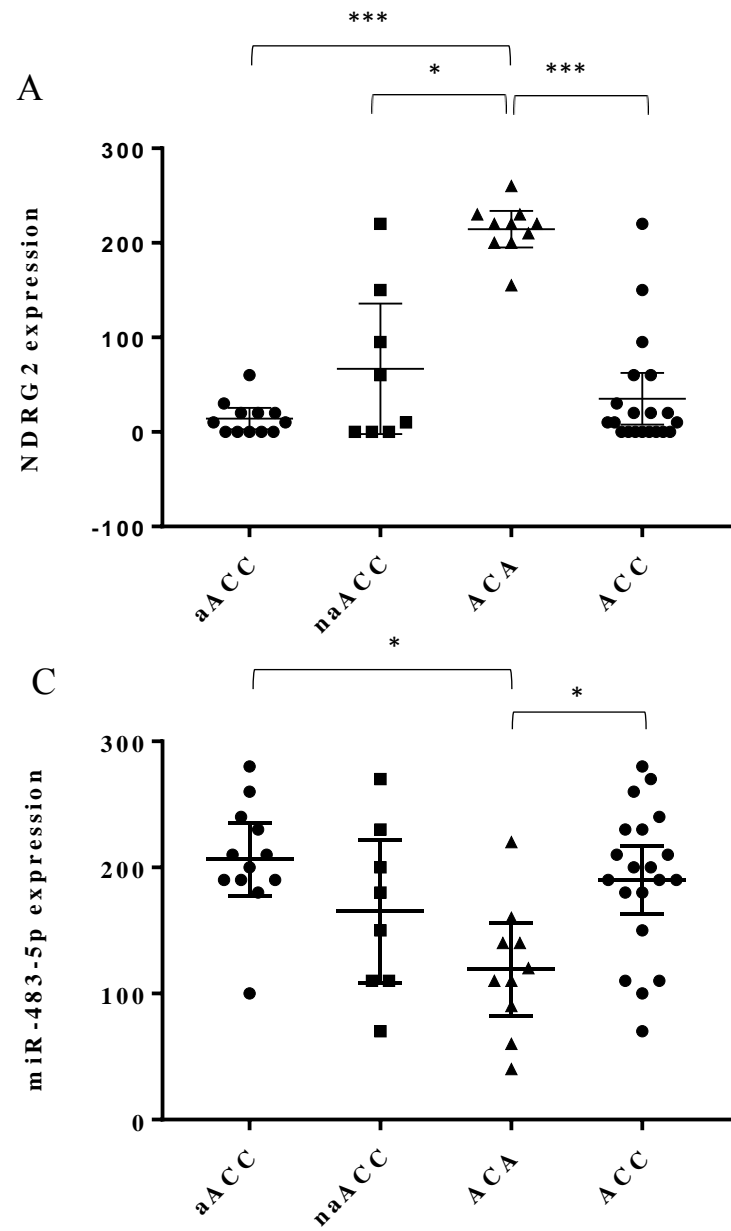


Figure 2. NDRG2 (A), NDRG4 (B) and miR-483-5p (C) expressions in adrenocortical tumors by immunochemistry and *in situ* hybridization. Statistical analysis was performed using Kruskal-Wallis test for unpaired data and $p < 0,05$ was considered significant (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

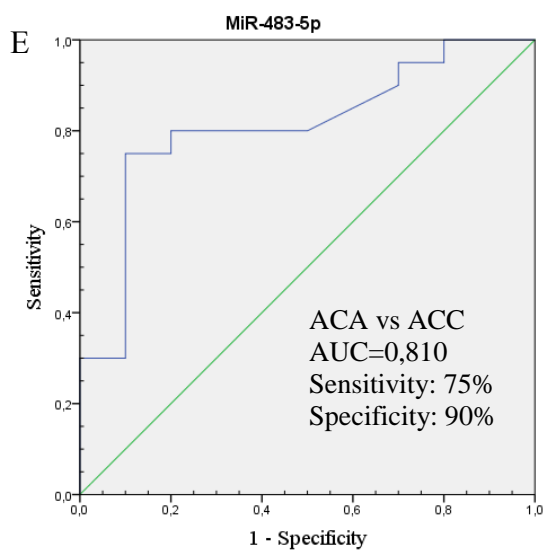
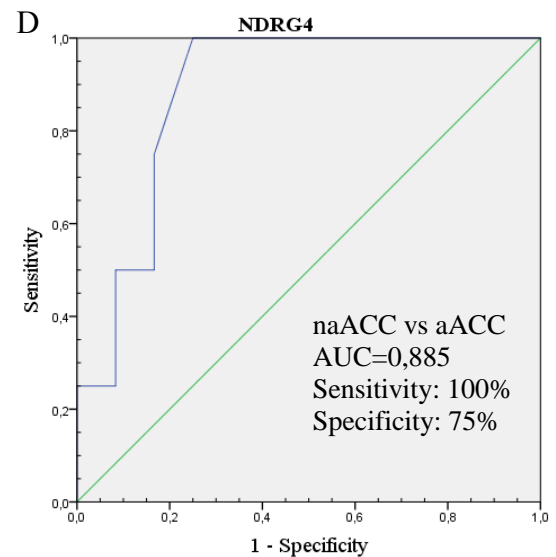
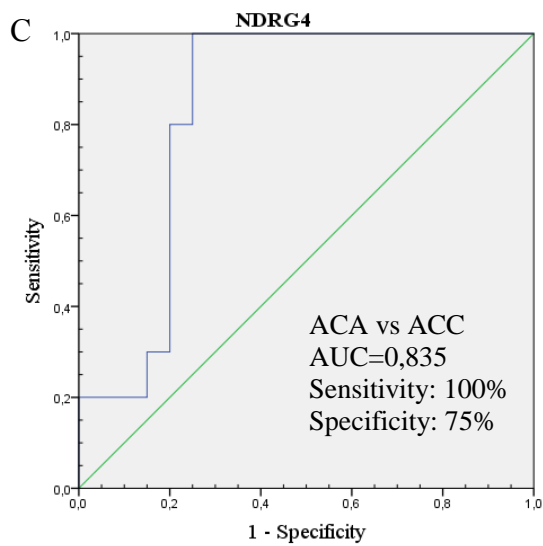
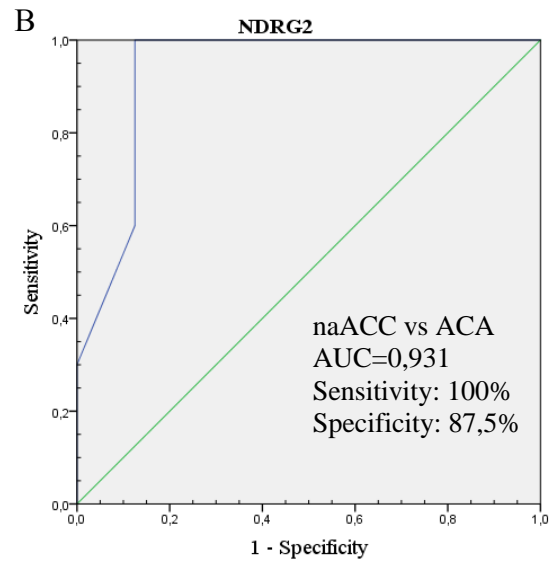
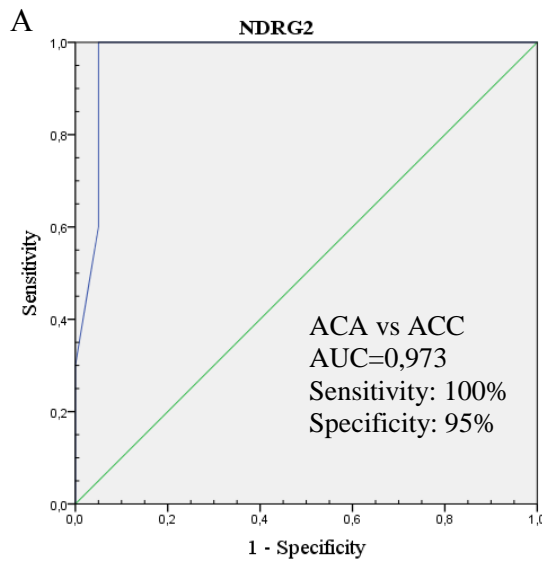


Figure 3. Diagnostic values of NDRG2, NDRG4 and miR-483-5p scores. ROC curves analyses of NDRG2, NDRG4 and miR-483-5p expression for discrimination between ACA and ACC (A, C and E respectively). (B) ROC curves analyses of NDRG2 expression for discrimination between naACC and ACA. (D) ROC curves analyses of NDRG4 expression for discrimination between naACC and aACC.

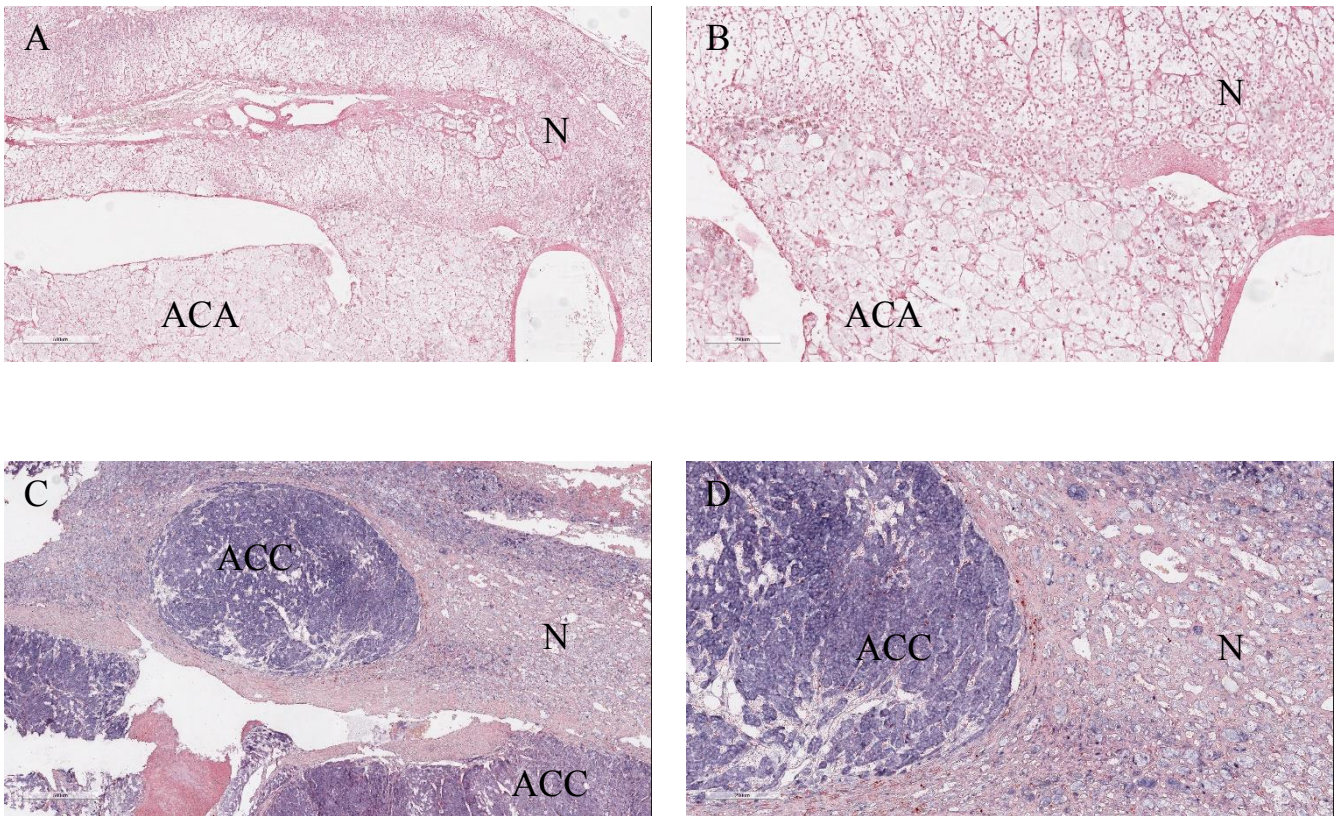


Figure 4. MiR-483-5p in situ hybridization in adenoma (A, B) and carcinoma (C, D). Adenoma (ACA) showed weak miR-483-5p expression, slightly similar to normal adrenal cortex (N), whereas carcinoma (ACC) demonstrated a strong cytoplasmic staining compared to normal adrenal cortex. Magnification x4 (A, C) and x10 (B, D).

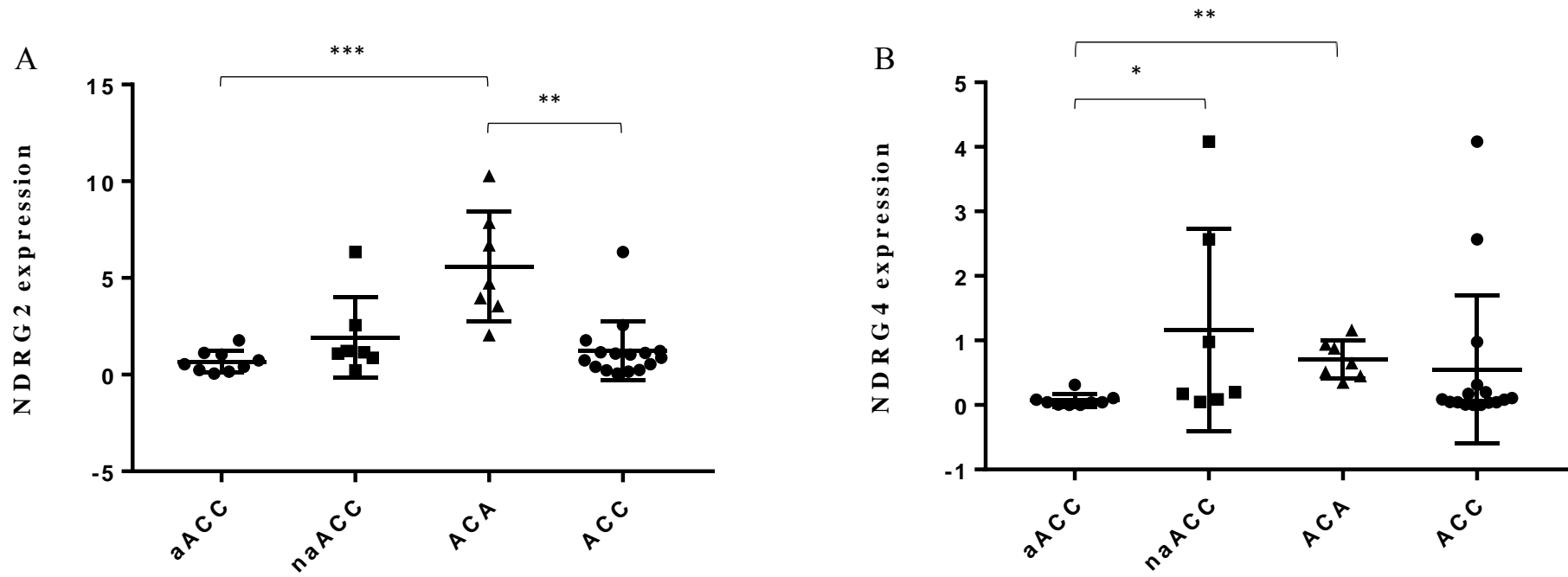


Figure 5. NDRG2 (A) and NDRG4 (B) expressions in adrenocortical tumors in RT-qPCR. Statistical analysis was performed using Kruskal-Wallis test for unpaired data and $p < 0,05$ was considered significant (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

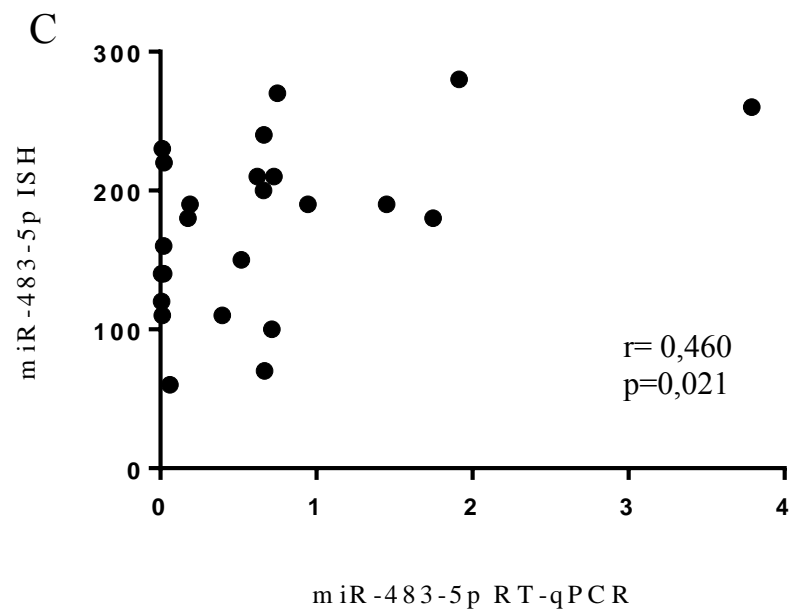
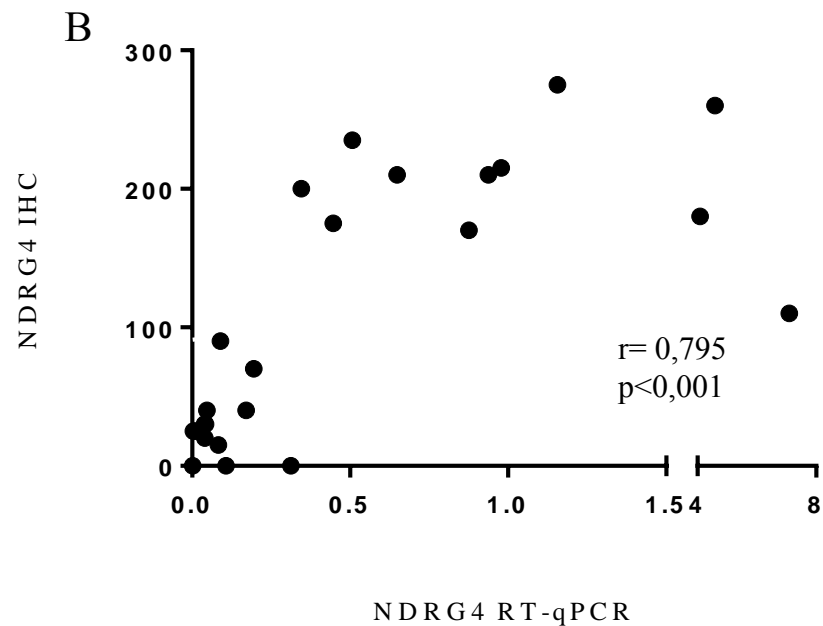
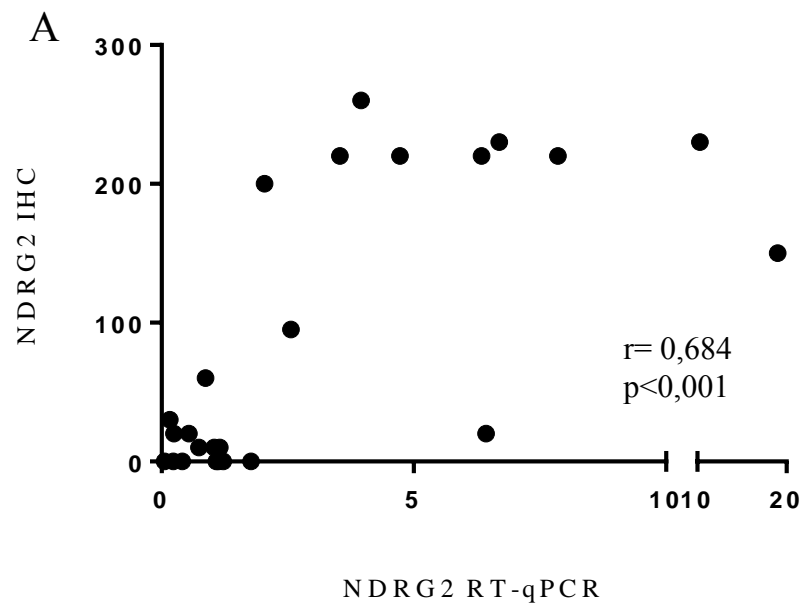


Figure 6. Correlation between NDRG2 (A) and NDRG4 (B) expression determined by quantitative RT-PCR analysis (RT-qPCR) and their scores determined by IHC, in adrenocortical tumors (ACA and ACC). (C) Correlation between miR-483-5p expression determined by RT-qPCR analysis and miR-483-5p score determined by ISH, in adrenocortical tumors.

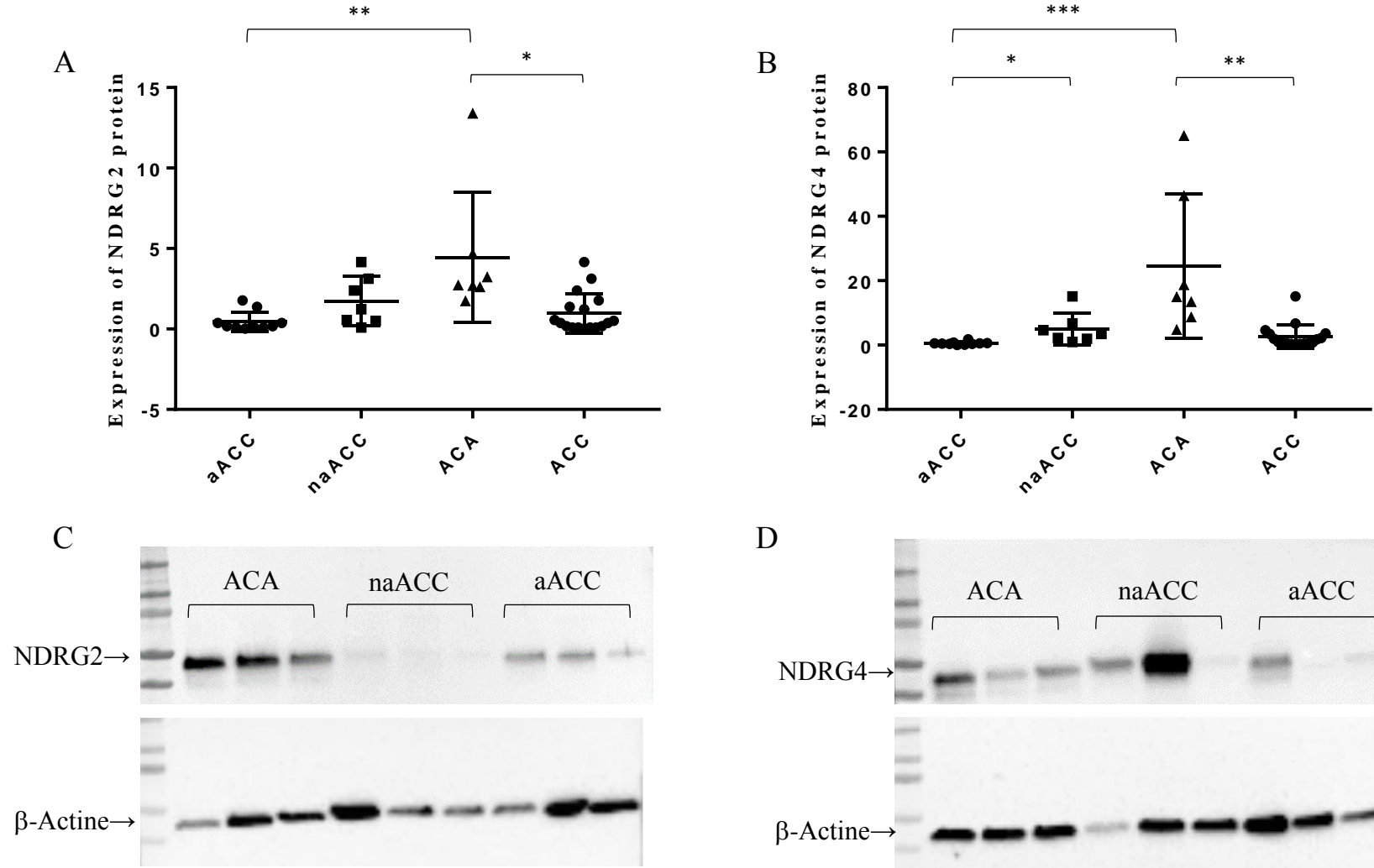


Figure 7. Protein levels of NDRG2 (A, C) and NDRG4 (B, D) determined by western blot analysis in adrenocortical tumors. β -Actine was used as a loading control. Statistical analysis was performed using Kruskal-Wallis test for unpaired data and $p < 0,05$ was considered significant (* $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$).

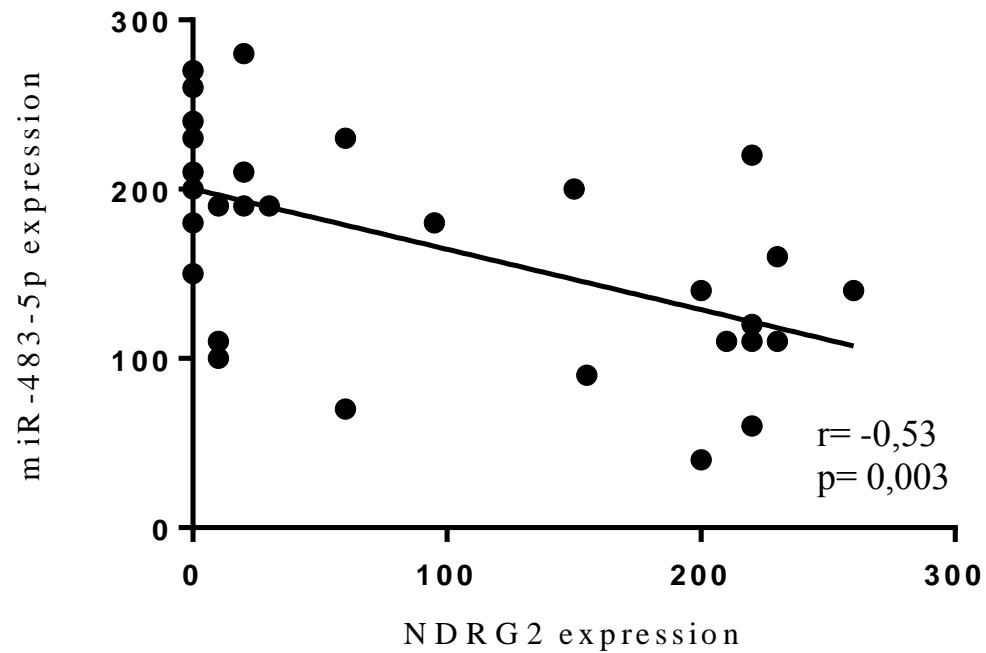


Figure 8. Correlation between miR-483-5p expression determined by *in situ* hybridization and NDRG2 expression determined by immunohistochemistry, in adrenocortical tumors (ACA and ACC).

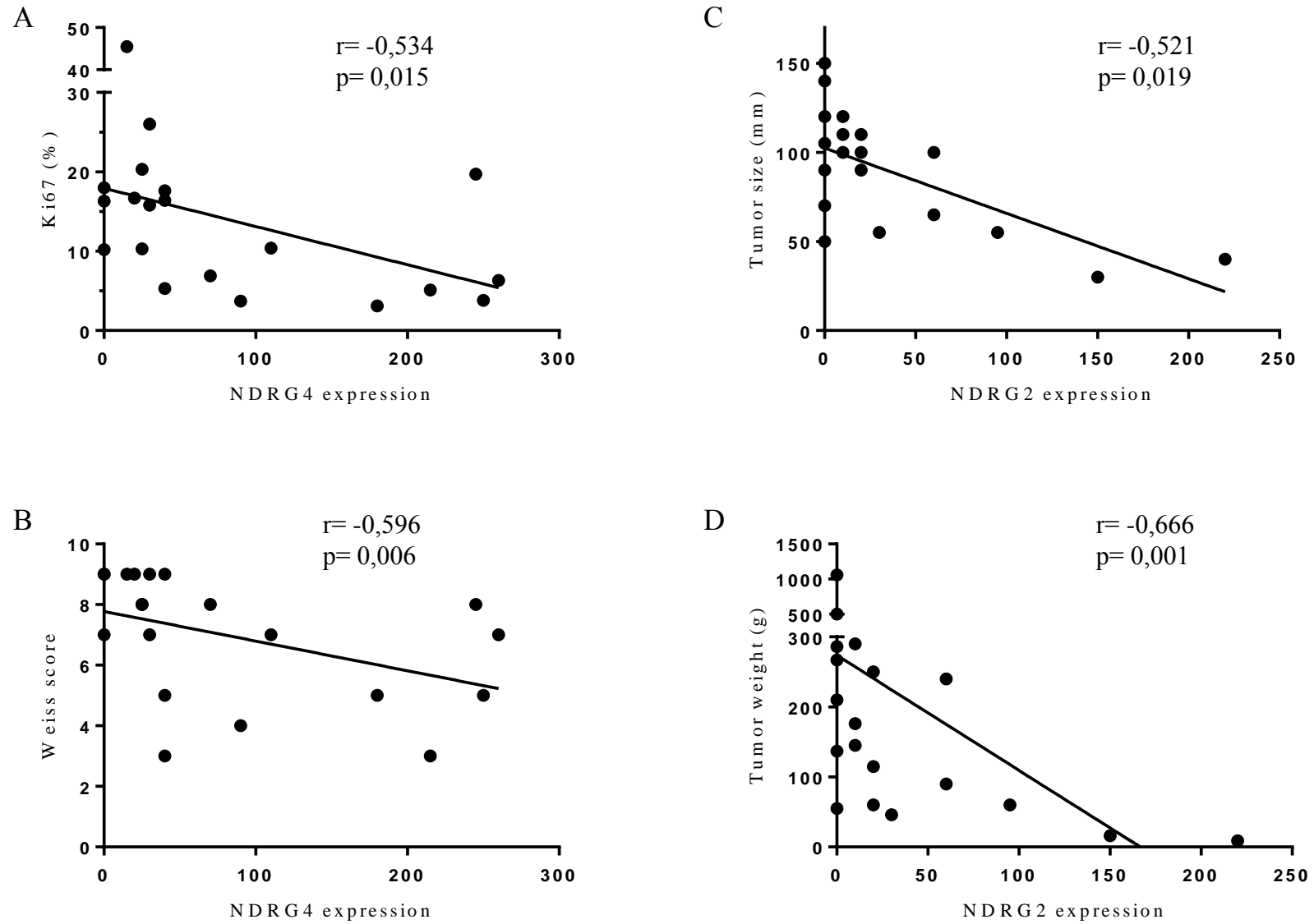


Figure 9. Correlations between NDRG4 and Ki67 (A) or Weiss score (B) and between NDRG2 and tumor size (C) or tumor weight (D) were analyzed using Spearman's test, in ACC.

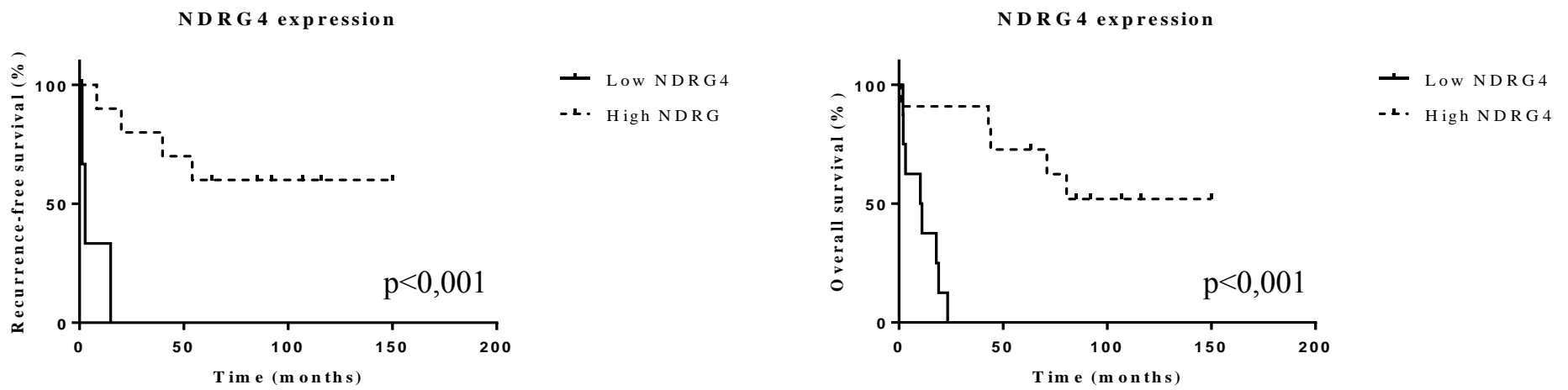


Figure 10. Kaplan-Meier analyses according to NDRG4 expression levels in ACC. (A) Recurrence-free survival as a function of NDRG4 levels. (B) Overall survival as a function of NDRG4 levels.

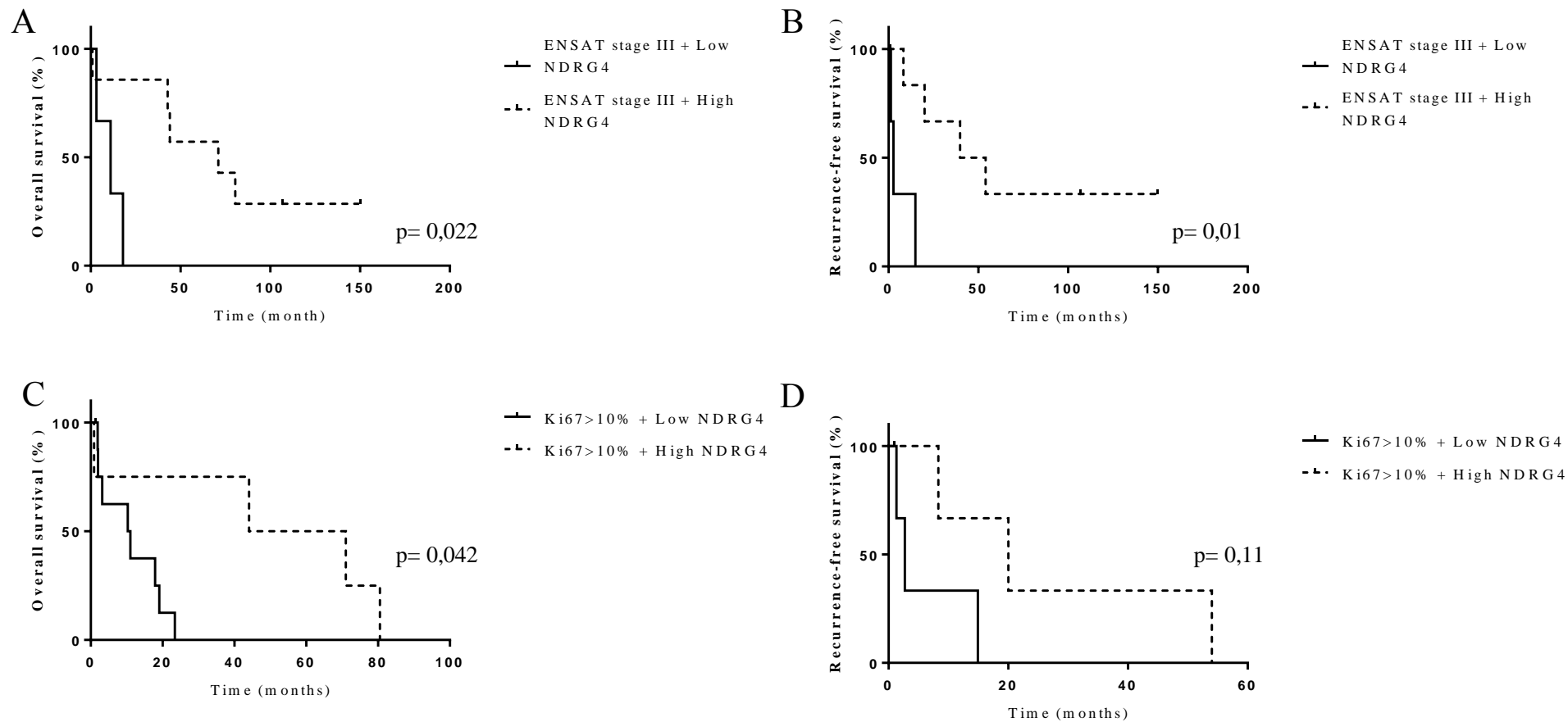
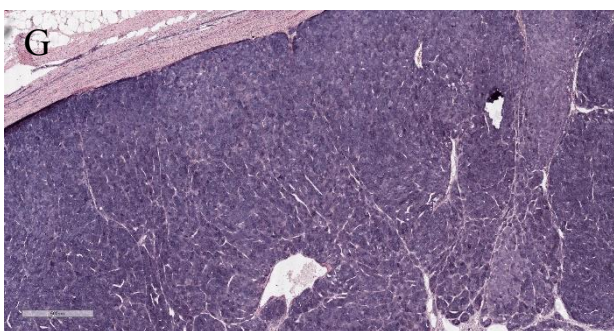
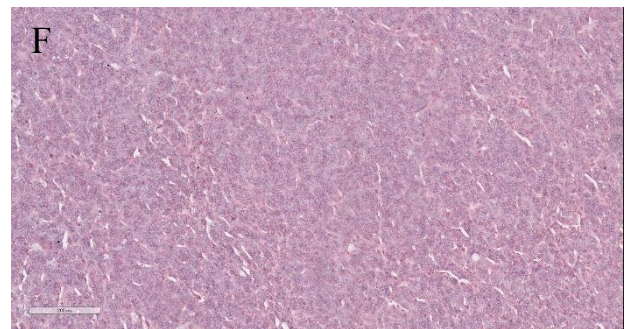
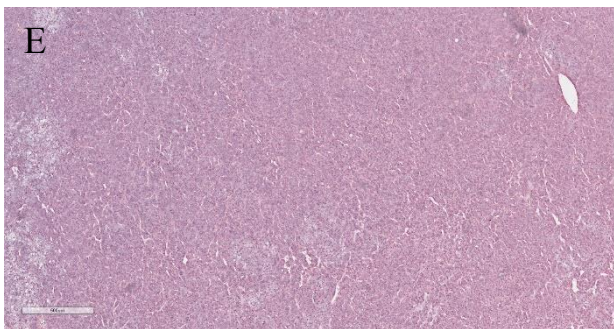
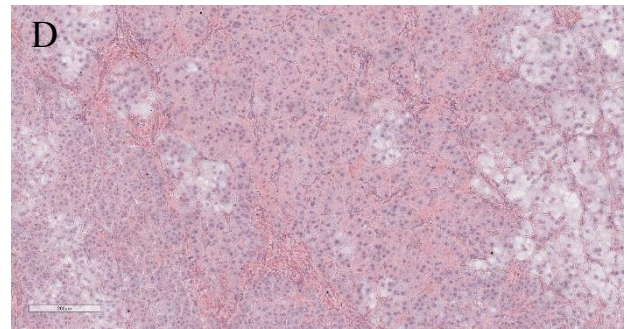
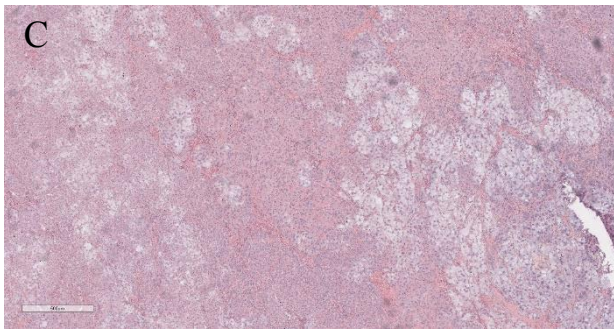
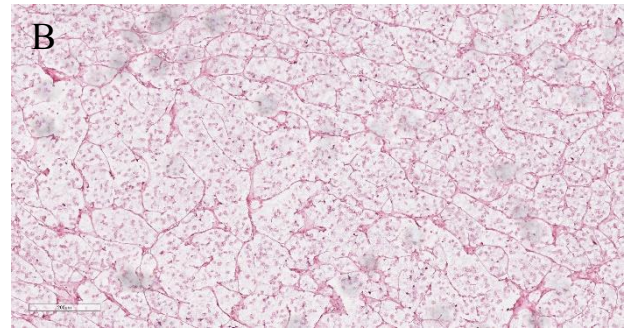
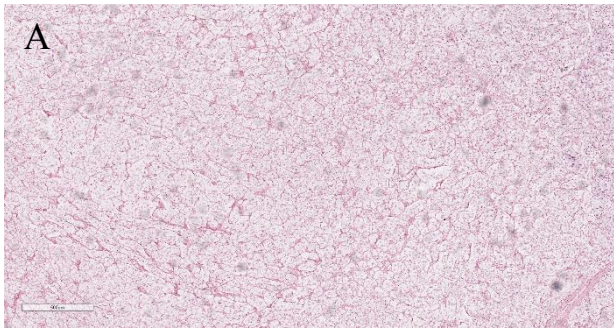


Figure 11. Prognostic values of ENSAT stage III combined with NDRG4 score, and Ki67>10% combined with NDRG4 score, in ACC. Kaplan-Meier estimates are presented for (A) overall survival and (B) recurrence-free survival for ENSAT stage III and (C) overall survival and (D) recurrence-free survival for Ki67>10%.



Supplementary Figure S1. Representative intensity levels for miR-483-5p detected by *in situ* hybridization. 0: complete absence of cytoplasmic or nuclear staining (A, B). 1: Weak staining often covering the nuclei (C, D). 2: moderate staining predominant in the cytoplasm (E, F). 3: Strong cytoplasmic staining (G, H). Magnification x4 (A, C, E) and x10 (B, D, F).

THÈSE SOUTENUE PAR : Bruno BOISSON

Intérêt diagnostique et pronostique de l'immunohistochimie NDRG2 et NDRG4 dans les tumeurs corticosurréaliennes

Le carcinome corticosurréalien (CCS) est un cancer rare et agressif, au pronostic péjoratif et aux options thérapeutiques limitées. Le diagnostic de malignité peut être difficile à établir pour le pathologiste et le pronostic des CCS reste incertain. MiR-483-5p et miR-139-5p sont sur-exprimés dans les CCS par rapport aux adénomes corticosurréaliens (ACS), et ciblent respectivement N-myc Downstream-Regulated Gene (NDRG) 2 et 4. Nous avons étudié l'expression de NDRG2 et NDRG4 en immunohistochimie (IHC) et de miR-483-5p et miR-139-5p en hybridation *in situ* (HIS) par des score semi-quantitatifs sur coupes de tumeurs corticosurréaliennes fixées dans le formol, en corrélation avec le niveau d'expression des gènes mesuré par RT-qPCR et le niveau d'expression des protéines mesuré par western blot sur tissus congelés. Dix ACS et vingt CCS ont été rétrospectivement sélectionnés (1994-2014). Les CCS ont été répartis en deux groupes, un groupe agressif (aCCS) et un groupe non-agressif (naCCS). L'expression de NDRG2 est significativement diminuée dans les CCS par rapport aux ACS ($p < 0.001$) et le score 152.5 correspond à la valeur seuil qui discrimine les ACS des CCS avec une sensibilité de 100% et une spécificité de 95%. L'expression de NDRG4 est statistiquement diminuée dans les aCCS versus les naCCS ($p = 0.043$) et un score inférieur à 35 permet de distinguer les aCCS avec une sensibilité de 100% et une spécificité de 75%. Nous avons mis en évidence une forte corrélation entre les scores semi-quantitatifs établis par IHC et l'expression des gènes en RT-qPCR et des protéines par western-blot. De plus, l'expression de NDRG2 mesurée par IHC est inversement corrélée à celle de son miARN régulateur détectée par ISH. En conclusion, notre étude confirme la sous-expression de NDRG2 et NDRG4, protéines cibles respectives de miR-483-5 et miR-139-5p, dans les CCS, par une technique immunohistochimique simple et un scoring semi-quantitatif utilisables en routine. Elle démontre également l'intérêt diagnostique de NDRG2 et pronostique de NDRG4 dans la prise en charge des CCS.

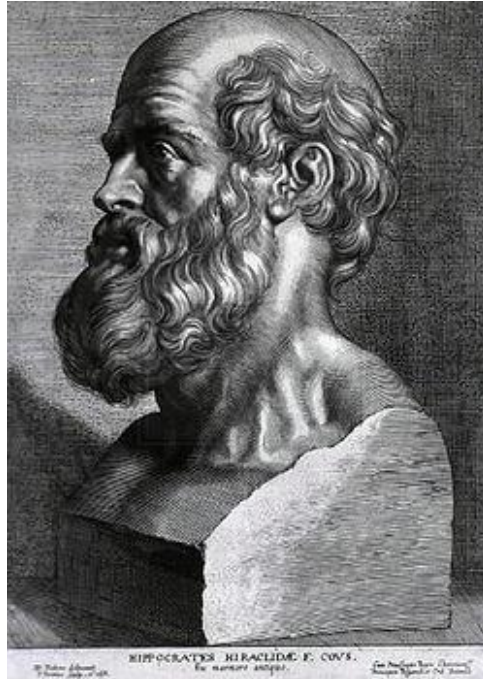
VU ET PERMIS D'IMPRIMER
Grenoble, le 22-09-2017

LE DOYEN
J.P. ROMANET

Pour la Présidente
et par délégation
Le Doyen de Médecine
Pr. Jean-Paul ROMANET

LE PRESIDENT DE LA THESE
PROFESSEUR N. STURM

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Tél. : 04 76 76 63 66



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 donnerais mes soins gratuitement à l'indigent et n'exigerais 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

Le carcinome corticosurrénalien (CCS) est un cancer rare et agressif, au pronostic péjoratif et aux options thérapeutiques limitées. Le diagnostic de malignité peut être difficile à établir pour le pathologiste et le pronostic des CCS reste incertain. MiR-483-5p et miR-139-5p sont sur-exprimés dans les CCS par rapport aux adénomes corticosurréaliens (ACS), et ciblent respectivement N-myc Downstream-Regulated Gene (NDRG) 2 et 4. Nous avons étudié l'expression de NDRG2 et NDRG4 en immunohistochimie (IHC) et de miR-483-5p et miR-139-5p en hybridation *in situ* (HIS) par des score semi-quantitatifs sur coupes de tumeurs corticosurréaliennes fixées dans le formol, en corrélation avec le niveau d'expression des gènes mesuré par RT-qPCR et le niveau d'expression des protéines mesuré par western blot sur tissus congelés. Dix ACS et vingt CCS ont été rétrospectivement sélectionnés (1994-2014). Les CCS ont été répartis en deux groupes, un groupe agressif (aCCS) et un groupe non-agressif (naCCS). L'expression de NDRG2 est significativement diminuée dans les CCS par rapport aux ACS ($p < 0.001$) et le score 152.5 correspond à la valeur seuil qui discrimine les ACS des CCS avec une sensibilité de 100% et une spécificité de 95%. L'expression de NDRG4 est statistiquement diminuée dans les aCCS versus les naCCS ($p = 0.043$) et un score inférieur à 35 permet de distinguer les aCCS avec une sensibilité de 100% et une spécificité de 75%. Nous avons mis en évidence une forte corrélation entre les scores semi-quantitatifs établis par IHC et l'expression des gènes en RT-qPCR et des protéines par western-blot. De plus, l'expression de NDRG2 mesurée par IHC est inversement corrélée à celle de son miARN régulateur détectée par ISH. En conclusion, notre étude confirme la sous-expression de NDRG2 et NDRG4, protéines cibles respectives de miR-483-5 et miR-139-5p, dans les CCS, par une technique immunohistochimique simple et un scoring semi-quantitatif utilisables en routine. Elle démontre également l'intérêt diagnostique de NDRG2 et pronostique de NDRG4 dans la prise en charge des CCS.