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Expression of Wnt and TGF- β pathway components and key adrenal transcription factors in adrenocortical tumors – association to carcinoma aggressiveness

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Abstract

Factors controlling benign and malignant adrenocortical tumorigenesis are largely unknown, but several mouse models suggest an important role for inhibin- α (INHA). To show that findings in the mouse are relevant to human tumors and clinical outcome, we investigated the expression of signaling proteins and transcription factors involved in the regulation of *INHA* in human tumor samples. Thirty-one adrenocortical tumor samples, including 13 adrenocortical carcinomas (ACCs), were categorized according to Weiss score, hormonal profile, and patient survival data and analyzed using immunohistochemistry and RT-PCR.

Expression of the TGF- β signaling mediator SMAD3 varied inversely with Weiss score, so that SMAD3 expression was lowest in the most malignant tumors. By contrast, SMAD2 expression was upregulated in most malignant tumors. Wnt pathway co-receptors LRP5 and LRP6 were predominantly expressed in benign adrenocortical tumors. In ACCs, expression of transcription factors GATA-6 and SF-1 correlated with that of their target gene *INHA*. Moreover, the diminished expression of GATA-6 and SF-1 in ACCs correlated with poor outcome.

We conclude that the factors driving INHA expression are reduced in ACCs with poor outcome, implicating a role for INHA as a tumor suppressor in humans.

Keywords

adenoma; carcinoma; inhibin; signaling pathway; transcription factor

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Introduction

Adrenocortical tumors (ACTs) affect approximately 5% of the general population according to autopsy studies [6]. The majority of the tumors are adrenocortical adenomas (ACAs). A subset of ACAs require surgery, most often due to their functional or hormone-secreting nature [4]. Adrenocortical carcinoma (ACC) is a rare disease, often presenting with metastases, with an incidence of approximately 1:1000000 and comprising 0.2% of all cancer deaths in the United States [32].

Several growth factor families and transcription factors, including the transforming growth factor beta (TGF- β) superfamily, wingless-related mouse mammary tumor virus integration site (Wnt) family, steroidogenic factor-1 (SF-1), and transcription factor GATA-6, are known to regulate adrenocortical development and function (reviewed in [5]). The same factors are often present in ACTs [14,29], but their potential interplay in these tumors is unknown. The intracellular mediators of these signaling pathways and transcription factors are known to bind one another [2,9,12], and interestingly, each of these factors induces the expression of inhibin-alpha (INHA) (8, 10–13), a subunit of the heterodimeric inhibin.

INHA is both a target gene for and an atypical or antagonistic member of the TGF- β growth factor superfamily [20]. The TGF- β family is a well-known component in many malignant processes, in some instances promoting and in others attenuating cancer progression [20]. The classical intracellular mediators of the TGF- β cascade are the SMAD proteins, of which family member 2 and 3 (SMAD2 and SMAD3) mediate the signal inducing *INHA* gene expression [13,25]. In addition to the TGF- β cascade, *INHA* expression is regulated by transcription factors such as SF-1 and GATA-6 [11,30]. GATA-6 is a zinc finger transcription factor expressed in the adrenal cortex throughout development and adulthood [15]. SF-1, a transcription factor that interacts with GATA proteins [12], is crucial for adrenocortical development and function [23]. SF-1 has also been shown to cooperate with beta-catenin (CTNNB1) in inducing the *INHA* promoter in adrenocortical cells [9]. CTNNB1 is a downstream mediator of the Wnt family of growth factors, important for both normal development and various cancers [22]. In the adrenal cortex, especially Wnt4 is developmentally critical [10,31]. Collectively, these studies suggest that the Wnt and TGF- β pathways, as well as the SF-1 and GATA transcription factors, take part in adrenocortical signaling and gene regulation. Presumably, there are several levels of interactions between these effectors also in ACTs.

The mouse adrenal cortex is remarkably different from its human counterpart both morphologically and functionally [5]. While molecular pathways and factors have been studied extensively in mouse adrenal development and tumors (reviewed in [5,16]), there is a need to address the interrelationship of these factors in human ACTs. We have now explored the significance of several growth and transcription factors in a series of human ACTs. Our results demonstrate a downregulation of activating cascades and transcription factors with a concomitantly diminished *INHA* expression in aggressive ACC.

Materials and Methods

Patients and human tissue samples

We studied ACT tissue samples of 31 patients treated in Helsinki University Central Hospital between 1981 and 1998 (Table 1). Samples were obtained from surgically removed adrenocortical tumors. Survival data and cause of death of the patients were obtained from the Finnish Population Registry and Statistics Finland, respectively. Postoperative follow-up was at least 5 years, with the last check-up on 6th November 2008. The tumor material has

been described previously [3]. The samples were categorized according to Weiss score [33,34] and subcategorized by hormonal status.

Immunohistochemistry results were assessed by light microscopy, and the proportion of positive tumor cells was scored from 0% to 100% by a consensus of H.P. and J.A. Results shown are rounded to the nearest 10%. For SMAD2, SMAD3, and CTNNB1, nuclear and/or cytoplasmic stain was recorded as intracellular accumulation and scored positive. For INHA cytoplasmic stain, and for low-density lipoprotein receptor-related protein 5 and 6 (LRP5 and LRP6), cytoplasmic/membranous stain was scored as positive. Immunohistochemistry results for SF-1 and GATA-6 have been published previously [14]. The study protocol was approved by the Ethics Committee of Helsinki University Central Hospital, and all work was conducted in accordance with the Helsinki declaration.

RT-PCR

Total RNA was isolated from frozen tissues as described previously [14]. Purified RNA (1 µg) was reverse-transcribed, and 2 µL of the reaction mixture was used for each PCR reaction. Agarose gel electrophoresis (2%) in the presence of ethidium bromide demonstrated a band of expected size for each of the primer pairs. The primers used were as follows: *SMAD2* (220 bp), F 5'-GTTCCTGCCTTTGCTGAGAC-3' and R 5'-TCTCTTTGCCAGGAATGCTT-3'; *SMAD3* (176 bp), F 5'-TGCTGGTGACTGGATAGCAG-3' and R 5'-CTCCTTGGGAAGGTGCTGAAG-3'; *CTNNB1* (530 bp), F 5'-GTTCGTGCACATCAGGATAC-3' and R 5'-CGATAGCTAGGATCATCCTG-3'; *LRP5* (438 bp), F 5'-GCAAGAAGCTGTACTGGACG-3' and R 5'-TGTTGCAGGCATGGATGGAG-3'.

Immunohistochemistry

The immunohistochemistry protocol used has been described previously [14]. Briefly, paraffin-embedded human tissue samples were deparaffinized and incubated with primary antibody. Detection was performed using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Diaminobenzidine (Sigma-Aldrich Co., St. Louis, MO) was used as chromogen, and the sections were counterstained with hematoxylin. Antibodies and dilutions used were as follows: GATA-6 IgG sc-9055 (1:50) (Santa Cruz Biotechnologies, Santa Cruz, CA), SF-1 IgG 06-431 (1:200) (Upstate Biotechnology, Lake Placid, NY), INHA IgG MCA951S (1:25) (AbB Serotec, Dusseldorf, Germany), SMAD2 IgG 51-1300 (1:100), SMAD3 IgG 51-1500 (1:500), and CTNNB1 IgG 18-0226 (1:500) (all from Zymed Laboratories, South San Francisco, CA), LRP5 IgG 38311 (1:200) (Abcam, Cambridge, MA), and LRP6 IgG 2560S (1:200) (Cell Signaling Technology, Inc., Danvers, MA).

Statistical analysis

Correlations between continuous variables were tested using nonparametric Spearman's rho, and the difference between two groups was tested using nonparametric Wilcoxon's test. Statistical analyses were performed using the JMP 7.0 software. A *p* value <0.05 was considered to be significant.

Results

mRNA for SMAD2, SMAD3, LRP5 and CTNNB1 is present in the majority of adrenal tumors

We first investigated the presence of mRNA for factors of interest in ACT samples and found that *CTNNB1* and *SMAD2* were present in all samples studied, whereas *SMAD3* and *LRP5* mRNA was evident in the majority of samples (Figure 1A). Notably, there was no

LRP5 mRNA in any of the three ACC samples. Unfortunately, the amount of RNA was too low to enable analysis of *LRP6* mRNA.

Wnt pathway co-receptors LRP5 and LRP6 are predominantly expressed in benign adrenocortical tumors

The results of the immunohistochemical investigation of 31 ACT samples are presented in Table 1. First, we investigated the expression of Wnt pathway components in adrenocortical tumors. Immunohistochemical analysis of Wnt pathway co-receptors LRP5 and LRP6 [7] revealed that expression of both co-receptors is more often seen in Weiss score 0–3 tumors than in malignant score 4–9 tumors ($p < 0.05$) (Figure 1A–C, E–H). We opted to study these co-receptors instead of the Frizzled family receptors also involved in adrenocortical Wnt pathway [28] since the presence of LRP5/6 in the receptor complex is thought to be more specific for canonical CTNNB1 mediated signaling [7]. In CTNNB1 staining, no difference was detected between benign and malignant tumors (Figure 1D). Expression of LRP5 was more often found in virilizing tumors ($n=5$) than in all other tumor types ($n=26$) ($p < 0.05$), irrespective of whether the tumors were benign or malignant. LRP6 expression did not differ between tumor subtypes, suggesting that LRP5 and LRP6 are not fully interchangeable in human adrenocortical tumor signaling.

SMAD3 expression is associated with a benign phenotype in adrenocortical tumors

Next, we examined whether the intracellular mediators of the TGF- β pathway, the SMAD proteins, are associated with the expression of Wnt pathway components and Weiss score in human adrenal tumors. We found SMAD3 cytoplasmic/nuclear expression to be positively correlated with LRP5 and LRP6 expression (Spearman's rank correlation coefficient (r) = 0.36 for LRP5, = 0.56 for LRP6; $p < 0.05$ for both), while SMAD2 was not. SMAD2 and SMAD3 were also observed to differ in relation to Weiss score; SMAD2 expression directly correlated and SMAD3 inversely correlated with Weiss score (Figure 2). We conclude that the intracellular accumulation of SMAD3, like the expression of Wnt pathway co-receptors LRP5/6, is associated with more benignly behaving tumors.

We have previously shown SMAD3 to have a role distinct from SMAD2 in the activation of *INHA* promoter in ovarian granulosa cells [2]. As SMAD3 also seemed to be associated with another regulator of *INHA*, the Wnt pathway, we were prompted to investigate whether the expression of Wnt pathway components and *INHA* protein correlate with each other in adrenocortical tumors. We found *INHA* to significantly correlate with the Wnt pathway components LRP5 ($r = 0.79$), LRP6 ($r = 0.54$), and CTNNB1 ($r = 0.62$) ($p < 0.05$ for each). Thus, an interrelationship of the expression levels of *INHA* and the Wnt pathway components can be noted in human adrenocortical tumors.

Diminished GATA-6 and SF-1 correlate with poor outcome

The correlation of *INHA* with CTNNB1 was of interest, as the latter has been shown to interact with SF-1 and induce activation of the *INHA* promoter [9]. In addition, we have previously shown that GATA-4 interacts physically with SMAD3 to induce *INHA* promoter in granulosa cells [2]. For GATA-6, a similar cooperation with the TGF- β pathway in *INHA* activation was observed (Parviainen and Anttonen, unpublished data). These findings led us to hypothesize a role for SF-1 (in conjunction with GATA factors) in the regulation of *INHA* in human adrenocortical tumors. Using the same tumor material, we have previously shown that GATA-6 and SF-1 are predominantly expressed in benign tumors [14]. Having access to the survival data of the patients, we now investigated the role of these transcription factors relative to *INHA* in the malignant subgroup.

We found that INHA expression in carcinomas correlates with that of the major adrenal GATA factor GATA-6 ($r = 0.70$), and also with that of SF-1 ($r = 0.58$) ($p < 0.05$ for both). Among our material, ACC was lethal to more than 50% of the patients, consistent with known overall outcome [32], and in these aggressive carcinomas both GATA-6 and SF-1 expression was significantly diminished relative to the carcinomas with a favorable outcome ($p < 0.05$) (Figure 3).

Discussion

Growth factor cascades of adrenal cortex and adrenocortical tumors have been studied in mice, and knowledge of the role of *Inha*, *Smad*, *Ctnnb1*, and *Gata* protein-related signaling and gene regulation has been rapidly increasing based on studies utilizing various animal models (reviewed in [5,16]). Here, we present a series of human adrenocortical tumors and show that these previous findings in mice are reflected in human tumors and clinical outcome.

We observed diminished expression of transcription factors GATA-6 and SF-1 in more aggressively behaving ACC. This is consistent with our previous findings, linking low expression of these factors to a higher Weiss score [14]. GATA-6 and SF-1 are abundantly expressed in normal adrenal cortex [15,26], and we hypothesize that the more malignant carcinomas have lost the expression of these transcription factors regulating normal adrenal function. This finding is contrary to a recent report showing increased SF-1 expression in a significantly larger number of ACC samples [27]. There was, however, variation in the SF-1 expression levels between individual tumors [27]. Another study described decreased expression of SF-1 in ACCs relative to adenomas, and variability between different samples was also observed [18]. The varying expression levels in ACCs are probably dependent on regulators and factors yet to be discovered.

In childhood ACC amplification and overexpression of the *SFI* gene have also been detected [1,24]. The majority of our patients were adults, but when the results were analyzed by age, there was a statistically significant reduction in SF-1 expression with advancing age. In the three childhood ACCs in our series, SF-1 expression was, similarly to previous reports, relatively high (Table 1).

We observed that in human ACTs INHA expression correlated with that of GATA-6 and SF-1, suggesting that regulation of *INHA* gene in these neoplasms is at least partially dependent on these transcription factors. *Inha* knockout mice develop ACTs [21], suggesting that INHA is an essential factor in maintaining normal characteristics of adrenocortical tissue. Interestingly, when *Smad3* was also knocked out in the *Inha*^{-/-} mice, tumor progression was attenuated [19]. This contrasts with our finding of low SMAD3 expression being associated with a high Weiss score. However, within our human material, the closely related SMAD2 was highest in the most malignant tumors, and thus, might have a role similar to *Smad3* in knockout mice. We have previously shown that GATA-4/6 and SMAD3 cooperatively activate *INHA* in endocrine cells [2], and we hypothesize that GATA-6 and SMAD3 may together induce INHA expression in ACTs.

Wnt4 mRNA expression has recently been reported in human adrenal cortex and adrenocortical tumors, with high expression being seen especially in Conn's adenomas [17]. Here, we evaluated the expression of Wnt pathway co-receptors LRP5 and LRP6, essential for normal Wnt signaling [7], in ACTs and observed both to be expressed more frequently in low Weiss score tumors. The Wnt mediator CTNNB1 was in our material equally expressed in all tumors, in contrast to a French series [8,29]. Interestingly, SF-1, CTNNB1, and INHA expression was significantly correlated, suggesting a similar activating cooperation of SF-1

and CTNNB1 in the regulation of *Inha* as that reported earlier in rat adrenocortical cells [9]. In human ACTs, Wnt pathway mediators were also linked to the TGF- pathway based on expression patterns. We have analyzed fetal adrenals of *Wnt4* knockout mice [31] and have observed that Smad3 expression is enhanced relative to controls (Parviainen et al., unpublished data), suggesting that if Wnt4 signaling is disrupted, TGF- signaling is strengthened. This observation supports a link between these two signaling cascades in the adrenal cortex.

Based on the present results and earlier findings from several laboratories, we propose a model of signaling and gene regulatory components affecting *INHA* transcription in human ACTs (Figure 4). We hypothesize that in the most malignant carcinomas with poor outcome *INHA* transcription is attenuated as the intracellular and nuclear mediators SMAD3, GATA-6, and SF-1 are lost or diminished. It has to be emphasized, however, that functional studies on the interactions of these factors are ultimately needed to strengthen the presented model of their cooperation. The pathways associated with ACC aggressiveness found in the present study and earlier research may ultimately offer new diagnostic tools and potential therapeutic targets for the treatment of these neoplasms.

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References

1. Almeida MQ, Soares IC, Ribeiro TC, Fragoso MC, Marins LV, Wakamatsu A, Ressio RA, Nishi MY, Jorge AA, Lerario AM, Alves VA, Mendonca BB, Latronico AC. Steroidogenic factor 1 overexpression and gene amplification are more frequent in adrenocortical tumors from children than from adults. *J Clin Endocrinol Metab.* 2010; 95:1458–1462. [PubMed: 20080844]
2. Anttonen M, Parviainen H, Kyronlahti A, Bielinska M, Wilson DB, Ritvos O, Heikinheimo M. GATA-4 is a granulosa cell factor employed in inhibin-alpha activation by the TGF-beta pathway. *J Mol Endocrinol.* 2006; 36:557–568. [PubMed: 16720723]
3. Arola J, Liu J, Heikkilä P, Ilvesmaki V, Salmenkivi K, Voutilainen R, Kahri AI. Expression of inhibin alpha in adrenocortical tumours reflects the hormonal status of the neoplasm. *J Endocrinol.* 2000; 165:223–229. [PubMed: 10810286]
4. Bertherat J, Mosnier-Pudar H, Bertagna X. Adrenal incidentalomas. *Curr Opin Oncol.* 2002; 14:58–63. [PubMed: 11790982]
5. Bielinska M, Parviainen H, Kiiveri S, Heikinheimo M, Wilson DB. Review paper: origin and molecular pathology of adrenocortical neoplasms. *Vet Pathol.* 2009; 46:194–210. [PubMed: 19261630]
6. Brunt LM, Moley JF. Adrenal incidentaloma. *World J Surg.* 2001; 25:905–913. [PubMed: 11572032]
7. Cadigan KM, Liu YI. Wnt signaling: complexity at the surface. *J Cell Sci.* 2006; 119:395–402. [PubMed: 16443747]
8. Gaujoux S, Pinson S, Gimenez-Roqueplo AP, Amar L, Ragazzon B, Launay P, Meatchi T, Libe R, Bertagna X, Audebourg A, Zucman-Rossi J, Tissier F, Bertherat J. Inactivation of the APC gene is constant in adrenocortical tumors from patients with familial adenomatous polyposis but not frequent in sporadic adrenocortical cancers. *Clin Cancer Res.* 2010; 16:5133–5141. [PubMed: 20978149]
9. Gummow BM, Winnay JN, Hammer GD. Convergence of Wnt signaling and steroidogenic factor-1 (SF-1) on transcription of the rat inhibin alpha gene. *J Biol Chem.* 2003; 278:26572–26579. [PubMed: 12732619]

10. Heikkilä M, Peltoketo H, Leppaluoto J, Ilves M, Vuolteenaho O, Vainio S. Wnt-4 deficiency alters mouse adrenal cortex function, reducing aldosterone production. *Endocrinology*. 2002; 143:4358–4365. [PubMed: 12399432]
11. Ito M, Park Y, Weck J, Mayo KE, Jameson JL. Synergistic activation of the inhibin alpha-promoter by steroidogenic factor-1 and cyclic adenosine 3',5'-monophosphate. *Mol Endocrinol*. 2000; 14:66–81. [PubMed: 10628748]
12. Jimenez P, Saner K, Mayhew B, Rainey WE. GATA-6 is expressed in the human adrenal and regulates transcription of genes required for adrenal androgen biosynthesis. *Endocrinology*. 2003; 144:4285–4288. [PubMed: 12959982]
13. Kaivo-Oja N, Bondestam J, Kamarainen M, Koskimies J, Vitt U, Cranfield M, Vuojolainen K, Kallio JP, Olkkonen VM, Hayashi M, Moustakas A, Groome NP, ten Dijke P, Hsueh AJ, Ritvos O. Growth differentiation factor-9 induces Smad2 activation and inhibin B production in cultured human granulosa-luteal cells. *J Clin Endocrinol Metab*. 2003; 88:755–762. [PubMed: 12574210]
14. Kiiveri S, Liu J, Arola J, Heikkilä P, Kuulasmaa T, Lehtonen E, Voutilainen R, Heikinheimo M. Transcription factors GATA-6, SF-1, and cell proliferation in human adrenocortical tumors. *Mol Cell Endocrinol*. 2005; 233:47–56. [PubMed: 15767045]
15. Kiiveri S, Liu J, Westerholm-Ormio M, Narita N, Wilson DB, Voutilainen R, Heikinheimo M. Differential expression of GATA-4 and GATA-6 in fetal and adult mouse and human adrenal tissue. *Endocrinology*. 2002; 143:3136–3143. [PubMed: 12130579]
16. Kim AC, Barlaskar FM, Heaton JH, Else T, Kelly VR, Krill KT, Scheys JO, Simon DP, Trovato A, Yang WH, Hammer GD. In search of adrenocortical stem and progenitor cells. *Endocr Rev*. 2009; 30:241–263. [PubMed: 19403887]
17. Kuulasmaa T, Jaaskelainen J, Supola S, Pietiläinen T, Heikkilä P, Aaltomaa S, Kosma VM, Voutilainen R. WNT-4 mRNA expression in human adrenocortical tumors and cultured adrenal cells. *Horm Metab Res*. 2008; 40:668–673. [PubMed: 18553255]
18. Lefrançois-Martinez AM, Bertherat J, Val P, Tournaire C, Gallo-Payet N, Hyndman D, Veyssiere G, Bertagna X, Jean C, Martinez A. Decreased expression of cyclic adenosine monophosphate-regulated aldose reductase (AKR1B1) is associated with malignancy in human sporadic adrenocortical tumors. *J Clin Endocrinol Metab*. 2004; 89:3010–3019. [PubMed: 15181092]
19. Looyenga BD, Hammer GD. Genetic removal of Smad3 from inhibin-null mice attenuates tumor progression by uncoupling extracellular mitogenic signals from the cell cycle machinery. *Mol Endocrinol*. 2007; 21:2440–2457. [PubMed: 17652186]
20. Massague J, Blain SW, Lo RS. TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell*. 2000; 103:295–309. [PubMed: 11057902]
21. Matzuk MM, Finegold MJ, Mather JP, Krummen L, Lu H, Bradley A. Development of cancer cachexia-like syndrome and adrenal tumors in inhibin-deficient mice. *Proc Natl Acad Sci U S A*. 1994; 91:8817–8821. [PubMed: 8090730]
22. Nusse R. Wnt signaling in disease and in development. *Cell Res*. 2005; 15:28–32. [PubMed: 15686623]
23. Parker KL, Rice DA, Lala DS, Ikeda Y, Luo X, Wong M, Bakke M, Zhao L, Frigeri C, Hanley NA, Stallings N, Schimmer BP. Steroidogenic factor 1: an essential mediator of endocrine development. *Recent Prog Horm Res*. 2002; 57:19–36. [PubMed: 12017543]
24. Pianovski MA, Cavalli LR, Figueiredo BC, Santos SC, Doghman M, Ribeiro RC, Oliveira AG, Michalkiewicz E, Rodrigues GA, Zambetti G, Haddad BR, Lalli E. SF-1 overexpression in childhood adrenocortical tumours. *Eur J Cancer*. 2006; 42:1040–1043. [PubMed: 16574405]
25. Roh JS, Bondestam J, Mazerbourg S, Kaivo-Oja N, Groome N, Ritvos O, Hsueh AJ. Growth differentiation factor-9 stimulates inhibin production and activates Smad2 in cultured rat granulosa cells. *Endocrinology*. 2003; 144:172–178. [PubMed: 12488343]
26. Sasano H, Shizawa S, Suzuki T, Takayama K, Fukaya T, Morohashi K, Nagura H. Ad4BP in the human adrenal cortex and its disorders. *J Clin Endocrinol Metab*. 1995; 80:2378–2380. [PubMed: 7629233]
27. Sbiera S, Schmulz S, Assie G, Voelker HU, Kraus L, Beyer M, Ragazzon B, Beuschlein F, Willenberg HS, Hahner S, Saeger W, Bertherat J, Allolio B, Fassnacht M. High diagnostic and

- prognostic value of steroidogenic factor-1 expression in adrenal tumors. *J Clin Endocrinol Metab.* 2010; 95:E161–171. [PubMed: 20660055]
28. Suwa T, Chen M, Hawks CL, Hornsby PJ. Zonal expression of dickkopf-3 and components of the Wnt signalling pathways in the human adrenal cortex. *J Endocrinol.* 2003; 178:149–158. [PubMed: 12844346]
29. Tissier F, Cavard C, Groussin L, Perlemoine K, Fumey G, Hagnere AM, Rene-Corail F, Jullian E, Gicquel C, Bertagna X, Vacher-Lavenu MC, Perret C, Bertherat J. Mutations of beta-catenin in adrenocortical tumors: activation of the Wnt signaling pathway is a frequent event in both benign and malignant adrenocortical tumors. *Cancer Res.* 2005; 65:7622–7627. [PubMed: 16140927]
30. Tremblay JJ, Viger RS. GATA factors differentially activate multiple gonadal promoters through conserved GATA regulatory elements. *Endocrinology.* 2001; 142:977–986. [PubMed: 11181509]
31. Vainio S, Heikkila M, Kispert A, Chin N, McMahon AP. Female development in mammals is regulated by Wnt-4 signalling. *Nature.* 1999; 397:405–409. [PubMed: 9989404]
32. Wajchenberg BL, Albergaria Pereira MA, Medonca BB, Latronico AC, Campos Carneiro P, Alves VA, Zerbini MC, Liberman B, Carlos Gomes G, Kirschner MA. Adrenocortical carcinoma: clinical and laboratory observations. *Cancer.* 2000; 88:711–736. [PubMed: 10679640]
33. Weiss LM. Comparative histologic study of 43 metastasizing and nonmetastasizing adrenocortical tumors. *Am J Surg Pathol.* 1984; 8:163–169. [PubMed: 6703192]
34. Weiss LM, Medeiros LJ, Vickery AL Jr. Pathologic features of prognostic significance in adrenocortical carcinoma. *Am J Surg Pathol.* 1989; 13:202–206. [PubMed: 2919718]

Figure 1. Expression of Wnt- and TGF- related genes in human adrenocortical tumors
 RT-PCR using 14 adrenocortical tumor samples; the numbers indicate case # (see Table 1) and ACC depicts the patients with adrenocortical carcinoma (A). Expression of LRP5 (B, E and G) and LRP6 (C, F and H), as determined by immunohistochemistry, is significantly higher in Weiss score 0–3 tumors than in malignant Weiss score 4–9 tumors ($p < 0.05$). The circle indicates case #17 (corresponding immunohistochemistry in E–F) and the triangle case #26 (corresponding immunohistochemistry in G–H). -catenin nuclear/cytoplasmic expression (D) does not differ between the Weiss score groups. WS = Weiss score, ACA = adrenocortical adenoma, ACC = adrenocortical carcinoma.

Figure 2. Expression levels of TGF-β signaling proteins are associated with malignancy in adrenocortical tumors

Histological Weiss score 0–9 (0 is the most benign and 9 highly malignant) was used to define the malignancy of the tumors. SMAD2 (A, C, and E) immunohistochemical nuclear/cytoplasmic expression correlates positively ($r = 0.38$, $p < 0.05$) and SMAD3 (B, D, and F) negatively ($r = -0.47$, $p < 0.05$) with Weiss score in adrenocortical tumors ($n = 31$). The circle indicates case #1 (corresponding immunohistochemistry in C–D) and the triangle case #27 (corresponding immunohistochemistry in E–F). Weiss = Weiss score, ACA = adrenocortical adenoma, ACC = adrenocortical carcinoma.

Figure 3. Expression of GATA-6 and SF-1 according to outcome of adrenocortical carcinomas Nuclear expression of GATA-6 (A) and SF-1 (B) is significantly lower in fatal carcinomas (DOD, n=9) than in non-fatal carcinomas (NDOD, n=4) ($p < 0.05$). DOD = died of disease; NDOD = not died of disease.

Figure 4. Hypothetical drawing of molecular interactions affecting *INHA* gene expression in human adrenocortical neoplastic tissue

Wnt and TGF- β pathway signals are transmitted through cell surface receptors to intracellular mediator proteins. In the most malignant tumors, expression of SMAD3, LRP5/6, GATA-6 and SF-1 is diminished (dashed line) and the reading of the *INHA* gene is subsequently attenuated.

Table 1
Clinical and histopathological characteristics of adrenocortical tumors

Tissue samples were obtained from 31 patients, of which 18 patients had an adrenocortical tumor of Weiss score (WS) 0–3 and 13 patients had adrenocortical carcinoma (ACC). 9 of the ACC patients died of the disease (DOD). Samples were stained immunohistochemically for GATA-6, SMAD2, SMAD3, SF-1, -catenin, INHA, LRP5, and LRP6 proteins, and then studied microscopically.

Clinical Characteristics					Histopathological Characteristics									
#	age	g	DOD		WS	LRP5	LRP6	CTNNB1	SMAD2	SMAD3	INHA	GATA-6	SF-1	
WS 0-3	1	66	M	no	Nonfunctional	0	10	30	50	10	100	10	60	40
	2	53	F	no		1	10	10	60	10	90	10	90	10
	3	67	F	no		1	10	50	40	60	80	0	80	0
	4	71	F	no		0	0	0	10	10	50	0	10	0
	5	39	F	no	Conn's	1	50	50	40	100	90	20	50	50
	6	47	F	no		0	10	10	0	0	20	0	50	30
	7	69	M	no		0	30	30	60	30	40	20	20	0
	8	40	M	no		1	30	10	70	40	70	20	80	70
	9	65	M	no	Cushing's	1	20	10	70	10	90	10	80	50
	10	26	F	no		1	10	0	30	0	20	40	90	70
	11	54	F	no		1	30	40	60	50	100	50	80	10
	12	65	F	no		0	20	10	20	10	80	10	70	60
	13	39	F	no	Virilizing	0	40	50	90	40	70	60	20	30
	14	69	F	no		0	10	10	10	10	90	10	50	60
	15	47	M	no		2	40	60	50	10	100	80	0	0
	16	37	F	no		1	20	0	80	30	20	80	100	0
	17	51	F	no		3	50	60	40	10	60	70	50	50
	18	7	F	no		3	40	20	100	30	40	90	10	50
ACC	19	55	F	yes	Nonfunctional	9	10	10	60	20	40	10	0	10
	20	21	M	no		5	10	20	100	80	30	80	40	70
	21	65	M	yes		6	20	20	90	80	60	10	20	40
	22	69	M	yes		8	0	0	40	90	10	0	10	10
	23	76	M	yes		7	0	0	40	10	0	10	0	0
	24	68	M	yes		5	0	0	70	0	40	0	0	0

Clinical Characteristics				Histopathological Characteristics									
#	age	g	DOD		WS	LRP5	LRP6	CTNNB1	SMAD2	SMAD3	INHA	GATA-6	SF-1
25	76	F	yes	Conn's	6	0	0	10	20	80	0	0	0
26	50	M	yes		5	0	0	0	10	70	0	0	0
27	57	M	yes	Cushing's	8	0	0	10	80	0	0	0	10
28	1	F	no		4	0	0	30	30	10	0	30	20
29	39	F	no		6	20	0	30	30	10	30	50	10
30	74	F	yes	Virilizing	6	30	20	70	40	40	40	90	10
31	1	M	no		8	50	50	90	90	50	50	80	100

Results are presented as percentage of homogenous stain in the tumor cells, WS = Weiss score, ACC = adrenocortical carcinoma, g = gender, DOD = died of disease.p