Components of the Canonical and Non-Canonical Wnt Pathways Are Not Mis-Expressed in Pituitary Tumors

Leandro Machado Colli¹, Fabiano Saggioro², Luciano Neder Serafini², Renata Costa Camargo³, Helio Rubens Machado³, Ayrton Custodio Moreira¹, Sonir R. Antonini⁴, Margaret de Castro¹*

¹ Department of Internal Medicine, School of Medicine of Ribeirao Preto, University of Sao Paulo, Sao Paulo, Brazil, ² Department of Pathology, School of Medicine of Ribeirao Preto, University of Sao Paulo, Sao Paulo, Brazil, ³ Department of Surgery, School of Medicine of Ribeirao Preto, University of Sao Paulo, Sao Paulo, Brazil, ⁴ Department of Pediatrics, School of Medicine of Ribeirao Preto, University of Sao Paulo, Sao Paulo, Brazil

Abstract

Introduction: Canonical and non-canonical Wnt pathways are involved in the genesis of multiple tumors; however, their role in pituitary tumorigenesis is mostly unknown.

Objective: This study evaluated gene and protein expression of Wnt pathways in pituitary tumors and whether these expression correlate to clinical outcome.

Materials and Methods: Genes of the Wnt canonical pathway: activating ligands (WNT11, WNT4, WNT5A), binding inhibitors (DKK3, sFRP1), β-catenin (CTNNB1), β-catenin degradation complex (APC, AXIN1, GSK3β), inhibitor of β-catenin degradation complex (AKT1), sequester of β-catenin (CDH1), pathway effectors (TCF7, MAPK8, NFAT5), pathway mediators (DVL-1, DVL-2, DVL-3, PRICKLE, VANGL1), target genes (MYB, MYC, WISP2, SPRY1, TP53, CCND1); calcium dependent pathway (PLCB1, CAMK2A, PRKCA, CHP); and planar cell polarity pathway (PTK7, DAAM1, RHOA) were evaluated by QPCR, in 19 GH-, 18 ACTH-secreting tumors, 21 non-secreting (NS) pituitary tumors, and 5 normal pituitaries. Furthermore, no association of the studied gene or protein expression was observed with tumor size, recurrence, and progressive disease. The hierarchical clustering showed a regular pattern of genes of the canonical and non-canonical Wnt pathways randomly distributed throughout the dendrogram.

Results: There are no differences in gene expression of canonical and non-canonical Wnt pathways between all studied subtypes of pituitary tumors and normal pituitaries, except for WISP2, which was over-expressed in ACTH-secreting tumors compared to normal pituitaries (4.8x; p = 0.02), NS pituitary tumors (7.7x; p = 0.004) and GH-secreting tumors (5.0x; p = 0.05). β-catenin, NFAT5 and JNK proteins showed no expression in normal pituitaries and in any of the pituitary tumor subtypes. Furthermore, no association of the studied gene or protein expression was observed with tumor size, recurrence, and progressive disease.

Conclusions: Our data reinforce previous reports suggesting no activation of canonical Wnt pathway in pituitary tumorigenesis. Moreover, we describe, for the first time, evidence that non-canonical Wnt pathways are also not mis-expressed in the pituitary tumors.

Citation: Colli LM, Saggioro F, Serafini LN, Camargo RC, Machado HR, et al. (2013) Components of the Canonical and Non-Canonical Wnt Pathways Are Not Mis-Expressed in Pituitary Tumors. PLoS ONE 8(4): e62424. doi:10.1371/journal.pone.0062424

Edited by: Raul M. Luque, University of Cordoba, Spain

Copyright: © 2013 Colli et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by Sao Paulo Research Foundation (FAPESP grant numbers 2010/01286-7 and 2007/58365-3) and Brazilian National Council for Research and Development (CNPq grant number 2008/3003867). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: castrom@fmrp.usp.br

Introduction

Pituitary tumors cause significant morbidity by compression of central nervous system structures or inappropriate expression of pituitary hormones [1]. The molecular pathogenesis of sporadic or familial pituitary tumors remains mostly unknown; although, studies on genetic syndromes have provided new insights into the molecular basis of these tumors [1–6].

The Wnt pathway influences embryonic development, including axial disposition, organ formation, cell fate, and self-renewal of stem cells [7]. In physiological conditions, the Wnt pathway is activated when a Wnt ligand binds to its cell-surface receptor [8]. Ligand binding results in the dissociation of the β-catenin cytoplasmic degradation complex, comprising GSK-3β, APC, and AXIN1, resulting in β-catenin phosphorylation inhibition [7]. β-catenin thereby accumulates in the cytoplasm, translocates to the nucleus, and by binding to TCF/LEF, regulates the expression of several Wnt target genes, involved in cell growth and differentiation [8].

The Wnt signaling pathway has been implicated in the pathogenesis of several tumor types, such as colorectal [9], pediatric adrenocortical tumors [10], and craniopharyngioma [11]. In this latter, almost 80% of the cases of the adamantinomatous craniopharyngioma type showed aberrant cytoplasm and
nucleus β-catenin accumulation in contrary to the classical concentration in the cell membrane observed in normal tissue [12]. In addition, the prevalence of CTNNBI mutations in craniopharyngiomas observed in different series varies from 16 to 100% [11–14]. On the other hand, the role of Wnt pathway in pituitary tumors is still controversial in the literature. The initial work revealed nuclear β-catenin accumulation and suggested the involvement of the canonical pathway in pituitary tumorigenesis [15]. However, other studies failed to confirm the nuclear expression of β-catenin in large series of pituitary tumors [12,16,17]. Besides the canonical Wnt pathway, in which β-catenin is the central effector; there are the calcium-dependent and the planar cell polarity non-canonical Wnt pathways, which are β-catenin independent. Wnt binding to frizzled receptors signals to cell polarity and migration mediated by Disheveled (DVL) and JNK and to cell migration and invasion through stimulated calcium flux and activation of calcium-dependent enzymes calcium/calmodulin-dependent kinase II (CAMKII), calpain, and PKC [18,19]. Wnt can also signal in a β-catenin-independent fashion by binding to non-Frizzled receptors such as ROR2 [20]. There are relatively little understanding of the roles and the mechanisms of non-canonical Wnt pathways in tumorigenesis. Previous studies have implicated these pathways in cancer development [21–23]. Overexpression of Wnt5a is associated with migration and invasiveness in several cancers, including gastric and pancreatic as well as melanoma [24–26]. There are lacking studies addressing the involvement of the non-canonical Wnt pathways in the pathogenesis of the pituitary tumors.

In this context, the present study evaluates gene expression and the main effector proteins of canonical and non-canonical Wnt pathways in ACTH-, GH- secreting and non-secreting pituitary tumors to clarify their putative involvement in the pituitary tumorigenesis.

Materials and Methods
The study was approved by the Institutional Review Board of the University Hospital of the School of Medicine of Ribeirão Preto, University of Sao Paulo, Brazil (Process n° 8334/2005, 3608/2006, and 5283/2007). All participants gave written informed consent.

We studied 58 patients presenting pituitary tumors: 18 ACTH-, 19 GH-secreting and 21 non-secreting tumors. Table S1, Table S2, Table S3 show individual clinical and laboratorial features of the patients with different types of pituitary tumors.

All pituitary tumor samples were collected during transphenoidal surgery. Part of the tumor tissue was paraffin embedded for histopathological examination and the other part was microdissected by experienced pathologist to separate tumor from non-tumoral tissues. The microdissected tumor tissue was disrupted using a Polytron homogenizer and kept at −70°C for molecular studies. The control group comprised five normal pituitary tissues obtained during autopsies from subjects who had died due to cardiovascular acute disease, without previous evidence of any endocrine disease.

Tumoral total RNA were isolated by TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA). Sample integrity was evaluated by spectrophotometry at an absorbance of 260/280 nm and by agarose gel electrophoresis. cDNA were obtained using High capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA).

The relative expression of some genes of the Canonical Wnt pathway (WNT11, WNT4, WNT3a, DKK3, sFRP1, CTNNBI, APC, AXIN1, GSK3β, AKT1, CDH1, and TCF7), Calcium/Wnt pathway (PLCB1, CAMK2A, PRKCA, CHF1, and NFAT5), and Planar cell polarity pathway (FRICKLE, VANGL1, DVL-1, DVL-2, DVL-3, PTK7, DAAM1, RHOA, and MAPK8) were selected by literature reviews and by the KEGG pathway [7,27,28]. Reactions were incubated in a 96-well optical plate at 95°C for 15sec and 60°C for 1 min. Gene expression was calculated by QPCR software [29], using efficiency of each reaction. Fold change was determined by the median of each gene expression observed in pituitary tumors related to the median of gene expression observed in normal pituitaries.

Immunohistochemistry (IHC) studies were performed in tumor samples after deparaffinization, rehydration, antigen retrieval in citrate buffer solution (pH 6.0) for 45 min, and incubation for 2 h at room temperature with primary antibodies for the main effectors of canonical (β-catenin - Santa Cruz SC-7963), planar cell polarity (JNK - Abcam AB18680) and Calcium dependent (NFAT5 - Abcam AB3446) Wnt pathways. In ACTH-secreting pituitary tumors, IHC was performed in nine of the 18 samples studied by qPCR and in seven other ACTH-secreting tumors. The avidin-biotin system (Vectastain Elite ABC Kit, Burlingame, CA) were used to signal detection. We used craniopharyngioma tissue as positive control for β-catenin antibody; for NFAT5 and JNK antibodies we used two positive samples from GH-secreting pituitary tumor tissues. Each sample was assigned in 10 random fields as percentage of stained cells: negative (less than 1%), +1 (weak, 1–10%), +2 (moderate, 10–50%), and +3 (strong, more than 50%). The expression of each gene in pituitary tumors and in normal pituitary samples is presented as mean, standard deviation, median, and range. Statistical analysis was performed using Mann-Whitney test or Kruskal-Wallis test for multiple continuous variables (post test of Nemey-Dunn), when appropriate. Multivariable regression was used for continuous variables and logistic regression for categorical variables, such as gender, age at diagnosis, tumor size, altered visual field, remission of the disease, and tumor progression. Fisher’s exact test was also used for comparison of categorical variables, such as staining intensity by IHC between tumors and normal pituitaries. We used Agnes method for hierarchical clustering for gene expression in each tumor subtype. Data were analyzed by the R Project Software [30]. Differences were considered significant at p<0.05.

Results
Table1 shows the expression of studied genes of the canonical and non-canonical Wnt pathways in ACTH-, GH-secreting, and also in normal pituitaries. There are no differences in the expression of canonical and non-canonical Wnt pathway genes between each pituitary tumor subtype and normal pituitaries, as well as among all pituitary tumor subtypes, except for WISP2 which was over-expressed in ACTH-secreting tumors compared to normal pituitaries (4.8x; p = 0.02), to non-secreting pituitary tumors (7.7x; p = 0.004) and to GH-secreting tumors (5.0x, p = 0.05).

There was no association between the expression of each studied gene and gender, age at diagnosis, tumor size, altered visual field, remission of the disease, or tumor progression in any subtype of the pituitary tumors, but VANGL1 which was positively
Table 1. Wnt Pathway gene expression in Pituitary Tumors.

| Gene   | ACTH-Secreting Tumor | GH-Secreting Tumor | Non-Secreting Tumor | Normal Pituitary | p-value Kruskal-Wallis |
|--------|-----------------------|--------------------|---------------------|------------------|-----------------------|
|        | Mean ± SD (median)    | Mean ± SD (median) | Mean ± SD (median)  | Mean ± SD (median) |                       |
| AKT1   | 1.45±0.38 (1.13-1.64) | 1.13±0.43 (1.08-1.64) | 1.21±0.73 (0.84-1.72) | 0.85±0.23 (0.60-1.00) | 0.84 |
| APC    | 1.05±0.91 (1.18-1.72) | 1.30±0.75 (1.30-1.00) | 1.35±0.93 (1.35-1.00) | 0.88 (0.36-2.74) | 0.99 |
| PRKCA  | 1.71±0.37 (1.20-4.32) | 0.98 (0.09-3.07) | 1.13 (0.68-2.94) | 0.91 (0.55-1.30) | 0.96 |
| NFAT5  | 1.16±0.43 (1.34-1.51) | 1.35±1.39 (1.35-1.39) | 0.97±0.29 (0.97-0.29) | 0.91 (0.56-1.30) | 0.84 |
| CAMK2A | 1.05±0.43 (1.06-0.43) | 1.06 (0.04-4.68) | 0.99 (0.01-4.19) | 0.63 (0.30-4.21) | 0.72 |
| CCND1  | 1.24±1.16 (1.06-1.61) | 0.79±0.95 (0.79-0.95) | 0.91 (0.33-3.57) | 0.64 (0.39-1.42) | 0.76 |
| CDH1   | 1.18±0.60 (1.00-0.60) | 1.64±2.12 (1.64-2.12) | 2.56±1.42 (2.56-1.42) | 0.98 (0.78-1.41) | 0.87 |
| CHP    | 1.24±0.96 (1.22-0.70) | 1.17±0.73 (1.06-3.20) | 0.96±0.16 (0.96-0.16) | 0.91 (0.78-1.21) | 0.97 |
| CTNNB1 | 1.22±0.79 (1.09-0.58) | 1.17±0.83 (1.17-0.83) | 0.84±0.42 (0.84-0.42) | 0.72 (0.34-3.98) | 0.97 |
| DAA1   | 1.29±0.79 (1.67-2.16) | 1.91±2.88 (1.91-2.88) | 1.02±0.25 (1.02-0.25) | 0.54 (0.17-3.37) | 0.78 |
| DKK1   | 1.21±0.69 (1.16-9.03) | 1.03 (0.05-7.56) | 0.66 (0.11-7.09) | 2.1 (1.13-4.34) | 0.77 |
| DVL-1  | 1.07±0.49 (1.26-0.63) | 1.18±0.87 (1.18-0.87) | 0.86±0.41 (0.86-0.41) | 0.59 (0.05-2.10) | 0.97 |
| DVL-2  | 1.11 (0.41-2.41) | 1.13 (0.38-1.96) | 1.00 (0.42-3.71) | 0.91 (0.51-1.28) | 0.87 |
| DVL-3  | 0.99 (0.31-3.36) | 1.18±0.43 (1.18±0.43) | 0.81±0.37 (0.81±0.37) | 0.29 (0.64-1.88) | 0.97 |
| GSK3B  | 1.12±0.49 (1.11-0.42) | 1.21±0.92 (1.21±0.92) | 0.97±0.08 (0.97±0.08) | 0.76 (0.39-2.52) | 0.97 |
| MAPK8  | 1.30±1.76 (1.17-0.57) | 1.34±1.15 (1.34±1.15) | 0.96±0.22 (0.96±0.22) | 0.34 (0.18-7.32) | 0.97 |
| MYB    | 2.02±4.17 (2.74±0.95) | 7.82±32.11 (7.82±32.11) | 0.81±0.39 (0.81±0.39) | 0.56 (0.41-2.41) | 0.97 |
| MYC    | 0.60 (0.10-17.69) | 0.28 (0-3.68) | 0.50 (0.00-147.89) | 0.66 (0.42-1.31) | 0.93 |
| NFAT5  | 1.56±0.35 (1.58±1.35) | 2.35±4.64 (2.35±4.64) | 1.20±1.10 (1.20±1.10) | 0.93 (0.17-8.31) | 0.76 |
| NFKB   | 0.97 (0.10-17.69) | 1.04 (0.11-4.90) | 1.05 (0.06-21.48) | 0.51 (0.31-2.76) | 0.97 |
| PRKCA  | 1.03±2.45 (1.17±0.61) | 1.28 (0.18-3.49) | 1.12 (0.94-1.72) | 0.95 (0.94-1.72) | 0.97 |
| PTK7   | 1.29±0.96 (1.26±0.70) | 1.89±2.80 (1.89±2.80) | 0.92±0.27 (0.92±0.27) | 0.90 (0.00-6.84) | 0.97 |
| RHOA   | 1.03 (0.06-6.84) | 1.27 (0.32-3.55) | 0.88 (0.06-12.60) | 0.88 (0.53-1.23) | 0.91 |
| RICKLE | 1.37 (0.06-8.46) | 1.39 (0.10-1.03) | 1.40±1.19 (1.40±1.19) | 0.91±0.38 (0.91±0.38) | 0.91 |
| PRKCA  | 1.11 (0.22-4.32) | 0.99 (0.10-4.09) | 0.88 (0.08-4.85) | 0.78 (0.61-1.54) | 0.82 |
| PTEN   | 1.84±2.77 (1.45±0.87) | 1.24±0.63 (1.24±0.63) | 0.93±0.29 (0.93±0.29) | 0.64 (0.22-4.32) | 0.97 |
| RHOA   | 1.11 (0.15-12.36) | 1.51 (0.05-3.31) | 1.21 (0.13-2.36) | 0.96 (0.53-1.29) | 0.97 |
| RICKLE | 1.05±0.32 (1.13±0.43) | 1.08±0.45 (1.08±0.45) | 0.98±0.23 (0.98±0.23) | 0.93 (0.61-1.64) | 0.97 |
| RICKLE | 1.06 (0.61-1.64) | 1.07 (0.44-1.82) | 0.95 (0.41-1.94) | 1.04 (0.72-1.29) | 0.97 |
There was no significant difference in NFAT staining among all pituitary tumors and normal pituitaries (Figure 2). We also found no expression of NFAT protein, the Wnt/calcium pathway effector, in normal pituitaries, GH-secreting, and non-secreting pituitary tumors; but a weak NFAT staining was observed in 18.8% of ACTH-secreting pituitaries, GH-secreting, and non-secreting pituitary tumors; but due to sample unavailability, we chose some of the most important genes involved in the different steps of the Wnt pathways that include for canonical: activating ligands (WNT11, WNT4, WNT5A), binding inhibitors (DKK3, sFRP1), β-catenin gene (CTNNB1), β-catenin degradation complex (APC, AXIN1, GSK3β), inhibitor of β-catenin degradation complex (AKT), sequester of β-catenin (CDH1), pathway effectors (TCF7, MAPK3, AKT1, AKT2), important genes involved in the different steps of the Wnt pathways are not mis-expressed in the pituitary tumors. The present study assessed the expression of several genes of the canonical and non-canonical Wnt pathway genes in normal pituitary and in pituitary adenomas (Figure 1). The gene expression observed in normal pituitaries and in all subtypes of secreting- and non-secreting pituitary tumors was randomly distributed throughout the dendrogram.

#### Discussion

The present study assessed the expression of several genes of the canonical and non-canonical Wnt pathways as well as the main protein effectors of these pathways in a subset of ACTH-, GH-secreting and non-secreting pituitary tumors, respectively, without difference among pituitary tumors and normal pituitaries (Figure 2). We also found no expression of NFAT protein, the Wnt/calcium pathway effector, in normal pituitaries, GH-secreting, and non-secreting pituitary tumors; but a weak NFAT staining was observed in 18.8% of ACTH-secreting adenomas. There was no significant difference in NFAT staining among all pituitary tumors and normal pituitaries (Figure 2).

### Table 1. Cont.

| Gene | ACTH-Secreting Tumor | GH-Secreting Tumor | Non-Secreting Tumor | Normal Pituitary | p-value |
|------|----------------------|--------------------|---------------------|------------------|---------|
|      | Mean ± SD            | Mean ± SD          | Mean ± SD           | Mean ± SD        | Kruskal-Wallis |
|      | median (range)       | median (range)     | median (range)      | median (range)   |          |
| SPRY1| 57.55 ± 234.97       | 1.52 ± 1.45        | 1.61 ± 1.65         | 0.84 ± 0.38      | 0.83    |
|      | (0.13-999.0)         | (0.12-5.71)        | (0.18-5.99)         | (0.35-1.25)      |         |
| TCF7 | 1.86 ± 2.55          | 1.66 ± 2.17        | 1.38 ± 1.48         | 4.97 ± 5.71      | 0.31    |
|      | (0.08-10.35)         | (0.20-9.93)        | (0.07-4.61)         | (0.28-14.58)     |         |
| TPS3 | 1.21 ± 0.69          | 1.33 ± 1.07        | 1.16 ± 0.91         | 1.19 ± 0.44      | 0.71    |
|      | (0.06-2.80)          | (0.04-4.4)         | (0.30-4.11)         | (0.65-1.83)      |         |
| VANGL1| 1.28 ± 0.96         | 1.32 ± 1.52        | 3.01 ± 5.83         | 0.99 ± 0.30      | 0.94    |
|      | (0.27-3.96)          | (0.14-7.05)        | (0.10-24.23)        | (0.49-1.26)      |         |
| WISP2| 1.34 ± 1.34          | 2.08 ± 5.42        | 0.21 ± 0.18         | 0.36 ± 0.44      | 0.01    |
|      | (0.03-4.59)          | (0-23.77)          | (0.00-5.56)         | (0.07-1.13)      |         |
| Wnt11| 1.85 ± 2.46          | 4.16 ± 7.82        | 1.69 ± 1.52         | 0.82 ± 0.13      | 0.91    |
|      | (0.07-9.99)          | (0.03-26.78)       | (0.08-5.57)         | (0.71-1.03)      |         |
| Wnt4 | 2.24 ± 2.67          | 2.46 ± 3.36        | 2.44 ± 3.83         | 3.55 ± 3.54      | 0.31    |
|      | (0.04-7.92)          | (0.30-4.43)        | (0.04-24.23)        | (1.11-9.25)      |         |
| Wnt5A| 1.99 ± 2.30          | 1.43 ± 1.24        | 3.27 ± 6.64         | 1.55 ± 1.20      | 0.76    |
|      | (0.01-11.18)         | (0.06-11.2)        | (0.01-14.92)        | (1.11-9.25)      |         |
| sFRP1| 3.32 ± 3.23          | 1.95 ± 3.74        | 14.25 ± 39.04       | 0.50 ± 0.41      | 0.30    |
|      | (0.05-11.06)         | (0.01-11.77)       | (0.01-179.33)       | (0.17-1.14)      |         |
| GUSβ | 1.01 ± 0.35          | 1.26 ± 0.487       | 1.14 ± 0.80         | 1.04 ± 0.24      | 0.59    |
|      | (0.41-1.72)          | (0.51-2.02)        | (0.50-4.26)         | (0.68-1.35)      |         |
| PGK1 | 1.13 ± 0.45          | 0.91 ± 0.30        | 1.19 ± 0.78         | 0.94 ± 0.17      | 0.98    |
|      | (0.06-4.88)          | (0.04-4.44)        | (0.38-3.88)         | (0.69-1.13)      |         |
| TBP  | 1.05 ± 0.25          | 1.06 ± 0.34        | 1.04 ± 0.30         | 1.12 ± 0.28      | 0.99    |
|      | (0.50-1.39)          | (0.49-1.65)        | (0.44-1.63)         | (0.68-1.54)      |         |

DOI:10.1371/journal.pone.0062424.t001

associated with bigger tumors (p = 0.04; logistic regression) in all subtypes of pituitary tumors. Using logistic regression analysis, non-secreting pituitary tumors, as a group, were also associated with bigger tumor size; these tumors have the chance to be larger than ACTH- (+2.3 cm; p = 0.03) and GH-secreting pituitary tumors (+1.0 cm; p = 0.06).
NFAT5), pathway mediators (DVL-1, DVL-2, DVL-3, PRICKLE, VANGLI), and target genes (MYB, MYC, WISP2, SPRY1, TP53, CCND1); for calcium pathway: PLCB1, CAMK2A, PRKCA, and CHP; and for planar cell polarity pathway: PTK7, DAAM1, and RHOA.

We found some individual variability in the gene expression of canonical and non-canonical Wnt pathways, probably due to individual tumor heterogeneity; which has been frequently observed in arrays from other types of tumors [31–34]. No differential expression of the studied genes was observed between normal pituitaries and each different subtype of pituitary adenomas, except for over-expression of WISP2 in ACTH-secreting pituitary tumor. WISP2, a tumor suppressor gene involved in attenuating tumor invasion [35,36], has a glucocorticoid-responsive region in its promoter [37]. The elevated glucocorticoid levels observed in ACTH-secreting pituitary tumors would, therefore, over activate the WISP2 transcription.

β-catenin protein expression was also not accumulated in the nucleus/cytoplasm of the pituitary tumor cells, confirming no activation of canonical Wnt pathway in these tumors. The first paper published on β-catenin protein expression in pituitary tumors found β-catenin nuclear accumulation in 21 out of 37 (57%) tumors [15]. However, our data are in agreement with four other studies that found only slight β-catenin staining (0 to 2% of the cells) in a total of 231 pituitary tumors [12,16,17]. It is important to point out that CTXNB1 mutations were not found in 154 pituitary tumors previously evaluated, supporting the lack of the involvement of canonical Wnt pathway in the pathogenesis of pituitary tumors [17]. This is the reason that, in the present study, we did not sequence any gene involved in the Wnt pathway.

In addition, for the first time, we evaluated non-canonical Wnt/ calcium and the Wnt/polarity pathways in secreting and non-secreting pituitary tumors. Our data on mRNA and protein expression demonstrate no evidence for the involvement of non-canonical pathways in each different subtype of the pituitary tumors. Previous study had suggested in GHoromas and TSHomas a potential activation of non-canonical Wnt/cell polarity pathway, via activation of Erk1/2 MAPK by Wnt4 signaling [38]. However, it is important to note that Erk1/2 signaling may be due to the activation of other pathways, such as mTOR [39] and TGFβ [40].

Furthermore, we observed no association of expression of the studied mRNAs or the three main protein effectors of canonical (β-catenin) and non-canonical Wnt pathways (JNK and NFAT) with tumor size, recurrence, and progressive disease, except for over-expression of VANGL1, which was associated to bigger tumors. Indeed, the suppression of VANGL1 expression has been associated in vitro to the inhibition of the hepatocellular carcinoma growth [41]. In addition, VANGL1 is important in the brain development and mutations in this gene have been associated to neural-tube defects [42]. Finally, we cannot exclude some deregulation at other genes or proteins. However, the absence of alterations in the mRNA expression and protein levels of main effectors of canonical (β-catenin), calcium dependent (NFAT5), and planar cell polarity (JNK) suggests that even a hypothetical deregulation of other pathways would not influence directly the expression of genes of the canonical and non-canonical Wnt pathways.

We also used a bioinformatics approach, the Agnes-algorithm, to perform a hierarchical clustering of canonical and non-canonical Wnt pathway genes. The dendrogram showed a regular pattern of genes randomly distributed. We could not find a regular pattern of gene expression that would suggest a deregulated gene expression in any specific subtype of pituitary tumor, confirming...
the putative no involvement of the Wnt pathways in the pathogenesis of the pituitary tumors.

Recently, an elegant study generates a mouse that expressed a degradation-resistant mutant form of β-catenin in early Rathke’s pouch progenitors of the Wnt/β-catenin pathway and demonstrates that β-catenin–accumulating cells formed clusters, which resemble human craniopharyngiomas. These tumors arise from activation of β-catenin in pituitary progenitors during embryogenesis, since these clusters expressed stemness cell markers such as SOX2, SOX9, and p27Kip1 [43]. Mutation in the exon 3 of the CTNNB1 gene that codifies the binding site for the degradation complex of the β-catenin molecule is one of the molecular findings associated with adamantinomatous craniopharyngiomas [11,12,16,44]. Taking together, our data demonstrate no abnormal pattern of cytoplasmatic/nuclear β-catenin distribution in pituitary adenomas in contrast to craniopharyngiomas. From embryogenesis to adulthood, the number of proliferating cells decreases progressively, whereas the number of differentiated cells increases [45]; since there is no activation of Wnt pathways in pituitary tumors, we suggest that these tumors in contrast to craniopharyngiomas might arise from differentiated cells.

In conclusion, our data provide evidence that besides canonical, also the non-canonical Wnt pathway genes are not mis-expressed in the pituitary tumorigenesis.

Supporting Information

Table S1 Clinical and Laboratory Features of ACTH-secreting Pituitary Tumors.
(DOCX)

Table S2 Clinical and Laboratory Features of GH-secreting Pituitary Tumors.
(DOCX)

Table S3 Clinical and Laboratory Features of non-secreting Pituitary Tumors.
(DOCX)

Table S4 Genes of canonical and non-canonical Wnt Pathways and the respective qPCR assay identification probes.
(DOCX)
Reference

1. Melhem S (2011) Pathogenesis of pituitary tumors. Nature reviews Endocrinol-
ology 7: 257–266.

2. Kalnas GA, Kola B, Borboli N, Morris DG, Guerguev M, et al. (2002) 
Sequence analysis of the PRKAR1A gene in sporadic somatotroph and other 
pituitary tumours. Clinical Endocrinology 57: 443–448.

3. Zhuang Z, Ezzat SZ, Vorstmeier AO, Weil R, Oldfield EH, et al. (1997) 
Mutations of the MEN1 tumor suppressor gene in pituitary tumors. Cancer 
research 57: 5466–5471.

4. Pellagato NS, Quintanilla-Martinez L, Siggellok H, Simonsen E, Bink K, et al. 
(2006) Germ-line mutations in p27Kip1 cause a multiple endocrine neoplasia 
syndrome in rats and humans. Proceedings of the National Academy of Sciences 
of the United States of America 103: 15550–15556.

5. Vierimaa O, Georgitsi M, Lehtonen R, Valthero P, Kakko A, et al. (2006) 
Pituitary adenoma predisposition caused by germline mutations in the AIP gene. 
Science (New York, NY) 312: 1228–1230.

6. Guerguev M, Grossman AB (2011) Pituitary tumors in 2010: a new 
therapeutic era for pituitary tumors. Nature reviews Endocrinology 7: 71–73.

7. Logan CY, Nisse R (2004) The Wnt signaling pathway in development and 
disease. Annual review of cell and developmental biology 19: 781–810.

8. Chen X, Yang J, Evans PM, Liu C (2008) Wnt signaling: the good and the bad. 
Acta biochimica et biophysica Sinica 40: 577–594.

9. Burgess AW, Faux MC, Layton MJ, Ramsay RG (2011) Wnt signaling and 
colon tumorigenesis—a view from the periphery. Experimental cell research 317: 
2749–2758.

10. Leaf LF, Merjemo LM, Ramalho LZ, Marinelli CE, Yunes JA, et al. (2011) 
Wnt/beta-catenin pathway deregulation in childhood adenocortical tumors. 
The Journal of clinical endocrinology and metabolism 96: 3106–3114.

11. Sekine S, Shibata T, Kokubu A, Morishita Y, Noguchi M, et al. (2002) 
Wnt4 expression in human pituitary adenomas regulates activation of the 
Wnt-signalling. Acta neuropathologica 119: 631–639.

12. Campanini ML, Colli LM, Paixao BMC, Cabral TPF, Amaral FC, et al. (2010) 
Expression of Wnt4 in human pituitary adenomas regulates activation of the 
Wnt-signalling. Acta neuropathologica 119: 631–639.

13. Vierimaa O, Georgitsi M, Lehtonen R, Vahteristo P, Kokko A, et al. (2006) 
Germ-line mutations in p27Kip1 cause a multiple endocrine neoplasia 
syndrome in rats and humans. Proceedings of the National Academy of Sciences 
of the United States of America 103: 15550–15556.

14. Semba S, Han SY, Kokubu A, Morishita Y, Noguchi M, et al. (2002) 
Wnt4 expression in human pituitary adenomas regulates activation of the 
Wnt-signalling. Acta neuropathologica 119: 631–639.

15. Katoh M (2005) WNT/PCP signaling pathway and human cancer (review). 
Annual review of cell and developmental biology 20: 781–810.

16. Enomoto M, Hayakawa S, Itsukushima S, Ren DY, Matsuo M, et al. (2009) 
Wnt5a is correlated with aggressiveness of gastric cancer by stimulating cell 
migration and invasion. Cancer research 69: 10439–10448.

17. Ripka S, Koenig A, Buchholz M, Wagner M, Sipos B, et al. (2007) WNT5A— 
target of CUTF1 and potent modulator of tumor cell migration and invasion in 
pancreatic cancer. Cancergenesis 28: 1178–1187.

18. Guerguev M, Grossman AB (2009) Pituitary gland and beta-catenin signaling— 
from ontogeny to oncogenesis. Pituitary 12: 245–255.

19. Kanehisa M, Goto S, Yano Y, Mamio M, Tanabe M (2012) KEGG for 
integration and interpretation of large-scale molecular data sets. Nucleic acids 
research 40: D109–114.

20. Pahinger S, Thallinger GG, Snajder R, Eichhorn H, Rader R, et al. (2009) 
QPCR: Application for real-time PCR data management and analysis. BMC 
biinformatics 10: 269.

21. R Development Core Team (2011) R: A Language and Environment for 
Statistical Computing. Available: http://www.r-project.org.

22. ZieglerLund V, Hoffmann C, Gutierrez B, Krehan A, Kauf S, et al. (n.d.) 
Heterogeneous expression of tumor-associated genes in disseminated breast 
cancer cells. Anticancer research 27: 1769–1776.

23. Annye MJ, Liu W, Engelmann JC, Nuhn P, Gurcal M, et al. (2013) DNA 
methylation alterations exhibit intradividual stability and interindividual 
herogeneity in prostate cancer metastases. Science translational medicine 5: 
160ra10.

24. Bittner M, Melzer P, Chen Y, Jiang Y, Seftor E, et al. (2000) Molecular 
classification of cutaneous malignant melanoma by gene expression profiling. 
Nature 406: 536–540.

25. Hübzer F, Brabletz T, Budeczky J, Pfeiffer S, Jung A, et al. (2007) Heterogeneous 
expression of Wnt/beta-catenin target genes within colorectal cancer. Interna-
tional journal of cancer journal international du cancer 121: 1941–1948.

26. Davies SR, Davies ML, Sanders A, Parr C, Torkington J, et al. (2010) 
Differential expression of the CCN family member WISP-1, WISP-2 and WISP- 
3 in human colorectal cancer and the prognostic implications. International 
journal of oncology 36: 1129–1136.

27. Banerje S, Dhar G, Haq I, Kambhampati S, Mehta S, et al. (2008) CCN5/ 
WISP-2 expression in breast adenocarcinoma is associated with less frequent 
progression of the disease and suppresses the invasive phenotypes of tumor cells. 
Cancer research 68: 7606–7612.

28. Ferrand N, Strager E, Redeulli G, Sabbah M (2012) Glucocorticoids induce 
CCN5/WISP-2 expression and attenuate invasion in oestrogen receptor-
negative human breast cancer cells. The Biochemical journal 447: 71–79.

29. Miyakoshi T, Takeda M, Kajiy A, Egashira N, Takekoshi S, et al. (2008) 
Expression of Wnt4 in human pituitary adenomas regulates activation of the 
beta-catenin-independent pathway. Endocrine pathology 19: 261–273.

30. Duong MT, Aki S, Wei G, Wingate HF, Liu W, et al. (2012) LMW-E/CDK2 
deregulates aracnoid morphogenesis, induces tumorigenesis, and associates with 
the activated b-Raf/ERK1/2-mTOR pathway in breast cancer patients. PLoS 
genetics 8: e1002538.

31. Miyata N, Azuma T, Hozawa S, Higuchi H, Yokoyama A, et al. (2012) 
Transforming Growth Factor beta and Ras/MEK/ERK Signaling Regulate the 
Expression Level of a Novel Tumor Suppressor Lefty. Pancreas.

32. Yasgu R, Hamamoto R, Furukawa Y, Okabe H, Yamamura T, et al. (2002) 
Isolation and characterization of a novel human gene, VANGL1, as a 
threatenic target for hepatocellular carcinoma. International journal of 
oncology 20: 1173–1178.

33. Kihara Z, Torban E, McDearmid MR, Reynolds A, Berghout J, et al. (2007) 
Mutations in VANGL1 associated with neural-tube defects. The New England 
journal of medicine 356: 1432–1437.

34. GastonMaucaut C, Andoniadou CL, Signore M, Jayakody SA, Charolidi N, et al. 
(2011) Increased Wingless (Wnt) signaling in pituitary progenitor/stem cells 
gives rise to pituitary tumors in mice and humans. Proceedings of the National 
Academy of Sciences of the United States of America 108: 11482–11487.

35. Howing S-L, Wu C-H, Cheng T-S, Sy W-D, Lin P-CR, et al. (2002) Differential 
expression of Wnt genes, beta-catenin and E-cadherin in human brain tumors. 
Cancer letters 194: 191–197.