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## Assessment of the role of transcript for GATA-4 as a marker of unfavorable outcome in human adrenocortical neoplasms

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### Abstract

**Background:** Malignant neoplasia of the adrenal cortex is usually associated with very poor prognosis. When adrenocortical neoplasms are diagnosed in the early stages, distinction between carcinoma and adenoma can be very difficult to accomplish, since there is yet no reliable marker to predict tumor recurrence or dissemination. GATA transcription factors play an essential role in the developmental control of cell fate, cell proliferation and differentiation, organ morphogenesis, and tissue-specific gene expression. Normal mouse adrenal cortex expresses GATA-6 while its malignant counterpart only expresses GATA-4. The goal of the present study was to assess whether this reciprocal change in the expression of GATA factors might be relevant for predicting the prognosis of human adrenocortical neoplasms. Since human adrenal cortices express luteinizing hormone (LH/hCG) receptor and the gonadotropins are known to up-regulate GATA-4 in gonadal tumor cell lines, we also studied the expression of LH/hCG receptor.

**Methods:** We conducted a study on 13 non-metastasizing (NM) and 10 metastasizing/recurrent (MR) tumors obtained from a group of twenty-two adult and pediatric patients. The expression of GATA-4, GATA-6, and LH/hCG receptor (LHR) in normal and tumoral human adrenal cortices was analysed using reverse transcriptase-polymerase chain reaction (RT-PCR) complemented by dot blot hybridization.

**Results:** Messenger RNA for GATA-6 was detected in normal adrenal tissue, as well as in the totality of NM and MR tumors. GATA-4, by its turn, was detected in normal adrenal tissue, in 11 out of 13 NM tumors, and in 9 of the 10 MR tumors, with larger amounts of mRNA found among those presenting aggressive clinical behavior. Transcripts for LH receptor were observed both in normal tissue and neoplasms. A more intense LHR transcript accumulation was observed on those tumors with better clinical outcome.

**Conclusion:** Our data suggest that the expression of GATA-6 in human adrenal cortex is not affected by tumorigenesis. GATA-4 expression is more abundant in MR tumors, while NM tumors express more intensely LHR. Further studies with larger cohorts are needed to test whether relative expression levels of LHR or GATA-4 might be used as prognosis predictors.

## Background

Malignant neoplasia of the adrenal cortex is a rare pathological condition accounting for 0.05% – 0.2% of all cancers [1]. These tumors are usually associated with unfavorable prognosis. Five-year survival rate of patients presenting with evidences of adjacent tissue invasion, lymph node infiltration or metastases is reported to range between 15 to 25% [2]. Distinction between localized tumors with malignant behavior and benign adrenocortical neoplasms, based on clinical presentation and imaging studies, can be difficult [3]. Direct histopathologic analysis of tumoral samples has been widely accepted as the best available diagnostic tool to differentiate metastasizing and recurring from non-metastasizing and non-recurring tumors [3]. The evaluation of combined histologic features and establishment of specific scores, as proposed by Weiss [4], poses, however, some technical difficulties, even for skilled pathologists. This is particularly true for criteria such as nuclear grade, venous or sinusoid invasion, and diffuse architecture [5]. It is usually difficult to interpret and manage patients with tumors presenting an intermediate Weiss score of 3, which is considered a threshold value for malignancy [6]. Although high mitotic rate and DNA ploidy index may correlate with poor survival, not a single histopathologic feature has proven to be a reliable predictor of recurrence and metastasis [6-8]. Also, Weiss classification tends to be inaccurate for tumoral behavior prediction in children [9,10].

In order to provide reliable markers to predict tumoral outcome, the prognostic relevance of a wide array of genetic, molecular and immunohistochemical markers has been tested [2,3,11]. Adrenocortical malignant conversion has been associated with coordinated changes of antigen expression profile, such as undetectable levels of cytokeratins combined with high levels of vimentin [12]. Increased immunostaining index of the Ki67 antigen, which is a proliferation-associated nuclear protein, and elevated telomerase activity have also been related to more unfavorable prognosis [11,13,14].

Chromosomal instability is frequently described in association with tumoral progression. Genetic instabilities such as gains in chromosome 3, 9 and X, as well as losses of heterozygosity of 11q13 (MEN-1 locus) [15,16], 17p13 [17,18] and 11p15 [19] have all been reported to be prognostically relevant for adrenocortical malignancy. The 11p15 region harbors genes such as IGF-II, H19, H-ras and p57<sup>KIP2</sup>. Overexpression of insulin-like growth factors I and II (IGF-I and IGF-II, respectively), of IGF-I receptor and of IGF-binding protein 2 has been implicated in tumor progression and acquisition of the malignant phenotype in adrenal cortex [20,21]. Mutations of the p53 tumor suppressor (TP53) gene have been associated with

hereditary (Li-Fraumeni syndrome) and sporadic adrenocortical tumors [22-25]. Unfortunately, none of these markers has been shown to be consistently reliable, since both conflicting results and overlaps between benign adrenal adenomas and carcinomas have been reported [20-23].

Expression of nuclear transcription factors has been recently studied, including the cAMP-dependent transcription factors CREM and CREB. Preliminary results, although limited by the number of cases, suggest that the lack of expression of CREB and inducible cAMP early repressors (ICERs) might be linked to a more severe outcome [26]. The role of members of another family of transcription factors – GATA family – in adrenocortical tumorigenesis has been assessed. This family of transcription factors constitute a group of 6 structurally related zinc finger proteins, that binds to conserved (A/T)GATA(A/G) sequences. Based on sequence homology and expression profile GATA proteins have been classified into two sub-families: the hematopoietic group and the cardiac group. The first group comprises GATA-1, GATA-2 and GATA-3, and are mainly expressed in hematopoietic stem cells. The second includes GATA-4, GATA-5 and GATA-6, which are expressed in heart, lung, gut epithelium, liver, yolk sac endoderm, gonads, adrenal cortex, hypothalamus and pituitary [27,28]. GATA factors play an essential role in the developmental control of cell fate, cell proliferation and differentiation, organ morphogenesis, and tissue-specific gene expression [29-31]. High GATA-4 expression has been suggested to promote cell proliferation through an anti-apoptotic effect in mouse and human granulosa cells [32]. A decrease in GATA-4 expression is associated with follicular atresia through programmed cell death [33]. Various tumors have been associated with high expression of GATA-4 like granulosa and theca cell tumors [28], endodermal sinus tumor [34], esophageal/gastric adenocarcinomas [35], Sertoli and Leydig cell tumors [36]. Involvement of GATA-4 has been reported in mouse adrenocortical tumorigenesis [37]. Normal postnatal mouse adrenals express abundant GATA-6 mRNA, but no or little GATA-4 [37]. An opposite expression pattern, however, has been reported in mouse adrenocortical tumors, where GATA-4 is up-regulated and GATA-6 down-regulated during tumorigenesis. Using immunohistochemical staining, up-regulation of GATA-4 was also demonstrated in three human adrenocortical carcinomas, but not in normal adrenal tissue, adenomas or pheochromocytomas [37].

In order to assess whether this change in the pattern of GATA expression could be used in prognostic prediction for human adrenocortical neoplasm, we analysed the expression of GATA-4 and GATA-6 in 13 non-metastasiz-

**Table 1: Clinical Data of Patients Presenting with Adrenocortical Tumor**

Patient	Gender	Age at Diagnosis (yr)	Follow-up (yr)	Clinical Presentation	Tumor Evolution	Weiss Score
01	M	8	7.58	V	NM	4
02	M	9	7.58	V	NM	4
03	F	44	6.92	C	NM	0
04	M	2	3.75	V	NM	5
05	F	33	6.42	C	NM	0
06	F	29	6.42	C	NM	3
07	F	40	7.33	NF	NM	1
08	F	4	6	V	NM	NA
09	F	28	6.42	C	NM	NA
10	F	27	6.42	H	NM	NA
11	F	2	8.17	C and V	NM	2
12	F	24	5.92	C	NM	1
13	F	46	10	C	NM	1
14	M	29	1 (†)	C	MR	8
15	M	30	0.83 (†)	F	MR	NA
16	F	18	1.17 (†)	C and V	MR	8
17	F	23	2 (†)	C and V	MR	6
18	F	32	2.33 (†)	V	MR	NA
19	F	29	3 (†)	V	MR	NA
20	F	22	1 (†)	C and V	MR	NA
21	F	9	1.17 (†)	C	MR	7
22	M	2	6.25	V	MR	7

**V:** androgen-producing, **C:** glucocorticoid-producing, **H:** aldosterone-producing, **NF:** nonfunctioning, **F:** estrogen-producing, **†:** deceased, **NM:** non-metastasizing, **MR:** metastasizing/recurring, **NA:** insufficient data for Weiss score calculation.

ing and 10 metastasizing/recurrent tumors obtained from 22 patients.

## Methods

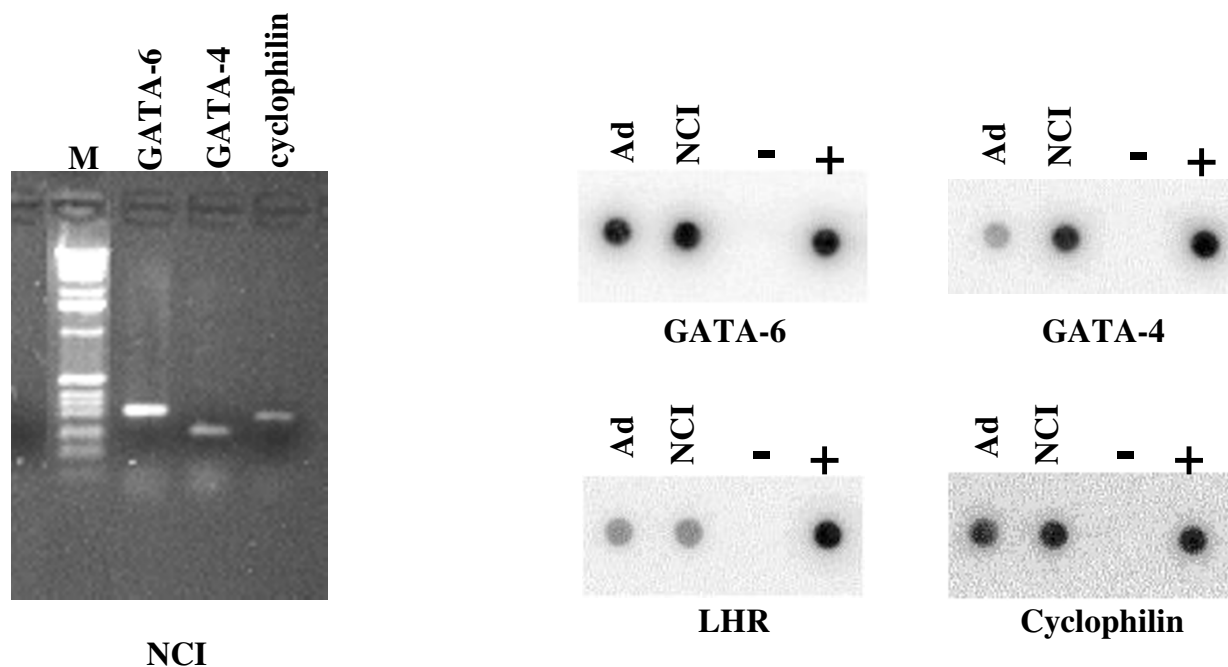
### Patients

Tissue specimens were obtained from twenty-two patients with adrenocortical tumors (Table 1). Most of them has been followed in our service since the initial diagnosis of adrenocortical neoplasm. A fraction of these patients were referred to us after undergoing adrenalectomy. Before surgery, informed consent was obtained from all patients or their parents. Ages varied from 2 to 46 years at the time of diagnosis, with 6 males and 16 females. Initial clinical presentation included Cushing's syndrome, adult female virilization, adult male feminization, hypertension, pseudoprecocious puberty, and only one patient with a non-functional tumor (Table 1). Based on their clinical behavior adrenal tumors were classified as nonmetastasizing (NM) or metastasizing/recurring (MR). Patients included in the NM group ( $n = 13$ ) presented no disease recurrence or metastasis after a mean follow-up of  $6.84 \pm 1.44$  years (Table 1). Conversely, except for patient 22, 9 patients in the MR group died within  $1.56 \pm 0.79$  years since the initial diagnosis, after presenting tumor dissemination and/or recurrence (Table 1). Patient 22, whose clinical data have been previously reported [25], presented two independent tumors excised from both left

and right adrenals in a two-year period. Subsequently solitary lung metastases were detected and surgically treated. He shows no signs of residual disease after a seven-year follow-up. A same pathologist reviewed histopathological data from all patients and, when sufficient data were available, calculated Weiss scores (Table 1).

### RNA extraction and cDNA synthesis

Tumor samples were immediately frozen in liquid nitrogen at the time of surgery and stored until RNA extraction. Total RNA from tumor samples and from human adrenocortical NCI-H295A cells was extracted using a guanidinium-thiocyanate-based commercial kit (TRI-Reagent, Sigma, St. Louis, MO). A commercial preparation of adult human adrenal total RNA (Human Adrenal Gland Total RNA, Clontech, Palo Alto, CA) was used as normal control. Five micrograms of total RNA from each sample were treated with 0.5 U of DNase I, (Promega Corp., Madison, WI) for 30 min at 37°C. DNase-treated RNAs were reversely transcribed at 42°C for 75 min with 200 U of a RNase H<sup>-</sup> MMLV reverse transcriptase (Superscript II, Life Technologies Inc. Gaithersburg, MD) in a final volume of 20 µl containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 1 mM of each dNTP, 100 mM dithiothreitol, 0.5 µg of oligo *dT* (Life Technologies Inc. Gaithersburg, MD), and 30 U of RNase inhibitor (RNasin, Promega Corp., Madison, WI).



**Figure 1**  
 RT-PCR and dot-blot hybridization for GATA-6, GATA-4, and LHR expression in normal human adrenal and in NCI-H295A cells. Correct sizes for GATA-6 and GATA-4 transcripts are, respectively, 285 and 194 bp. **M**: 1 kb molecular size ladder **Ad**: normal adrenal. **NCI**: NCI-H295A cells. +: genomic DNA. -: negative control for RT-PCR.

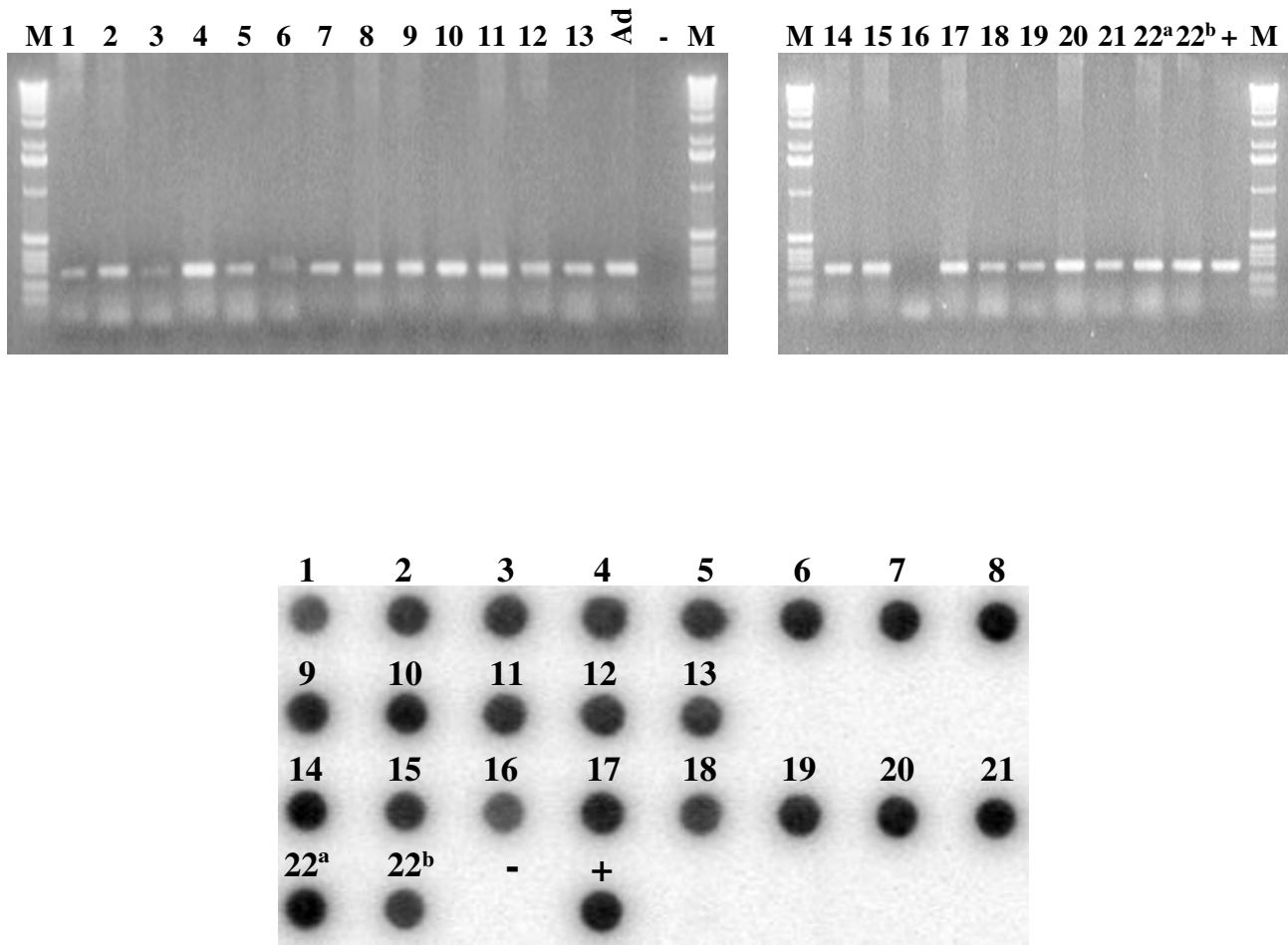
**Polymerase Chain-Reaction**

One tenth of RT products was amplified in a 50 µl reaction mix containing 20 pmol of each specific primer, 0.2 mM dNTP each, 50 mM KCl, 10 mM Tris-HCl (pH 9.3), 1.5 mM MgCl<sub>2</sub> and 5 U Taq Polymerase (Amersham-Pharmacia, Piscataway, NJ). Twenty microliters of the PCR product were loaded on a 1.5% agarose gel for electrophoresis and visualization with ethidium bromide. 194-bp GATA-4 and 285-bp GATA-6 cDNA fragments were amplified using oligonucleotides GATA4-F: 5'-TCCCTCT-TCCCTCCTCAAAT-3', GATA4-R: 5'-TCAGCGTGTAAG-GCATCTG-3', GATA6 - F: 5'-GAGAAGATGGAAGGGAAGGG-3', GATA6-R: 5'-TGTTG-TACCAAATGGCCTCAA-3', designed according to human GATA-4 and GATA-6 cDNA sequences (GenBank accession n° D78260 and U66075, respectively). Cycling conditions for PCR were: 95°C, 3 min, followed by 40 cycles at 95°C, 30 sec, 56°C, 30 sec, 72°C, 30 sec, and a final extension cycle of 7 min at 72°C. Amplification of a 234 bp fragment from the human luteinizing hormone receptor (LHR) transcript was accomplished using oligonucleotides eLHR: 5'-AGACATTCCAAAGAGATTC-3' and LHR2101R: 5'-GCAGTTACTGATGTAACAGTTAACAC-3'. Cycling conditions were essentially the same as those described for GATA cDNA amplification except for a 50°C

annealing temperature. As we amplified only exonic sequences, samples were tested with and without reverse transcriptase in order to rule out genomic DNA contamination. Samples quantity and integrity were verified by amplification of a 259-bp cyclophilin cDNA fragment using oligonucleotides CYC-F: 5'-TTTCACAGAAATTAT-TCCAGGGTTT-3' e CYC-R: 5'-CAATATTCATGCCTTCTT-TCACTT-3'. Direct sequencing was performed to confirm the identity of RT-PCR products.

**Dot-blot**

Fifteen microliters of GATA-4, GATA-6, LHR and cyclophilin PCR products were mixed with 185 µl NaOH 0.4 M, EDTA 25 mM, heated 95°C for 2 min, and vacuum-transferred onto a nylon membrane (Hybond N+, Amersham, Piscataway, NJ). Probes were generated by radioactive PCR as follows: fragments corresponding to GATA-4, GATA-6, LHR, and cyclophilin were amplified by PCR using genomic DNA as template. The amplification conditions and primer sets were the same as those described in the previous section. These fragments were then cloned into the pCR2.1-TOPO T.A. cloning vector (TOPO TA Cloning kit, Invitrogen, Carlsbad, CA). The identity of the desired cloned inserts was verified by direct sequencing. A second PCR was performed in a 25 µl reaction mix con-

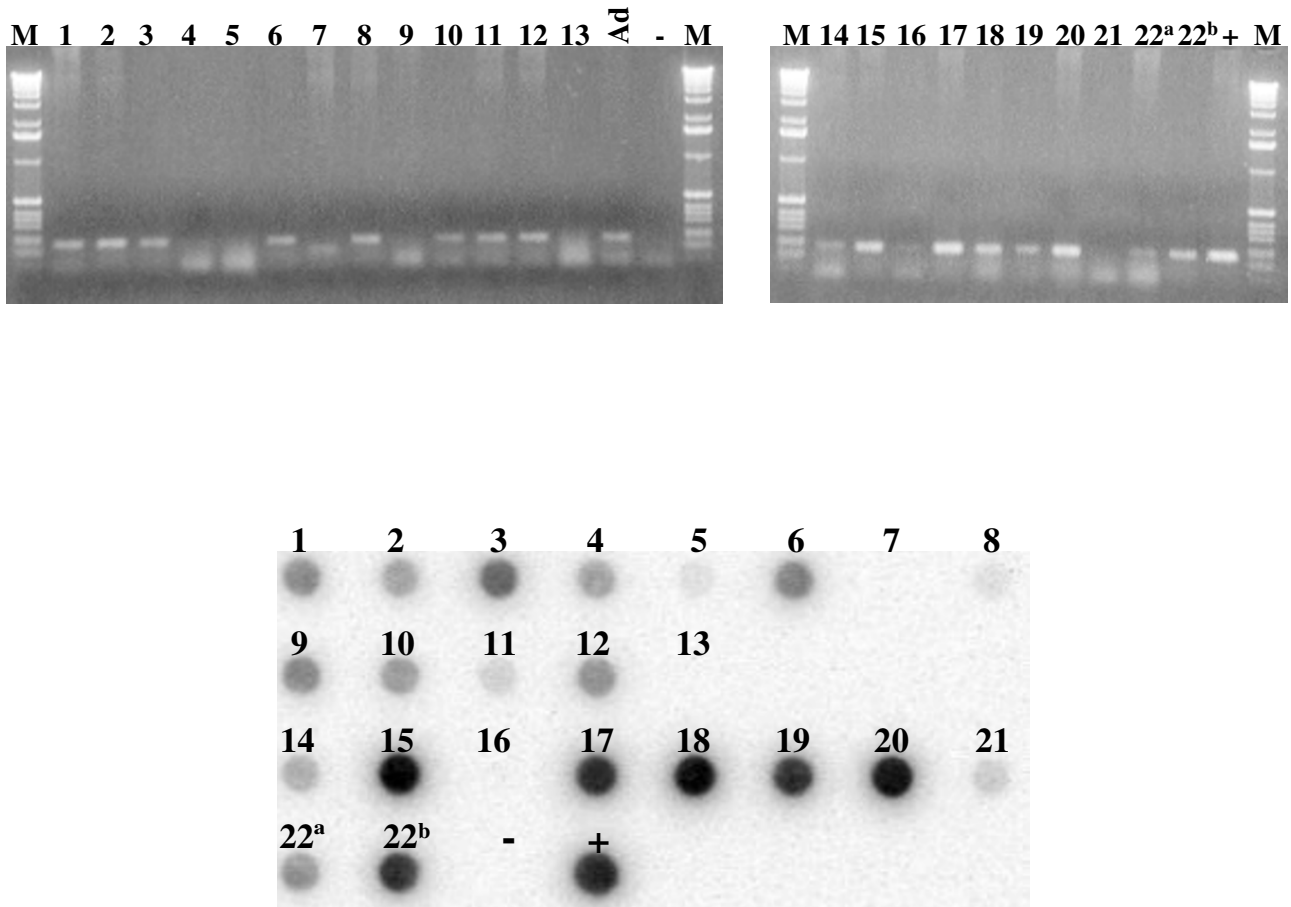


**Figure 2**

Expression of GATA-6 in neoplastic human adrenal tissues was evaluated by RT-PCR and dot-blot hybridization. RT-PCR products were resolved in 2% agarose gels. Tissue samples represented here, as well as in Figures 3–5, correspond to patients listed in Table 1. Samples 22a and 22b are tumor specimens removed from left (22a) and right (22b) adrenal of the same patient (patient 22, Table 1). **M**: 1 kb molecular size ladder **Ad**: normal adrenal. +: genomic DNA. -: negative control for RT-PCR. Samples 1 to 13: non-metastasizing; samples 14 to 22: metastasizing/recurring adrenocortical tumors.

taining 100 ng of plasmid (GATA-4-pCR2.1, GATA-6-pCR2.1, LHR-pCR2.1, and cyclophilin-pCR2.1), 20 pmol of M13F/R primers, 0.1 mM dNTP each, 50 mM KCl, 10 mM Tris-HCl (pH 9.3), 1.5 mM MgCl<sub>2</sub>, 0.5 µl of [ $\alpha$ -<sup>32</sup>P] dCTP (3000 Ci/mmol), and 5 U Taq Polymerase (Amersham-Pharmacia, Piscataway, NJ). Cycling conditions were: 94°C, 3 min, followed by 30 cycles at 94°C, 30 sec, 55°C, 30 sec, 72°C, 30 sec, and a final extension cycle of 7 min at 72°C. Probes were purified using Quiaquick PCR Purification kit (Quiaquick PCR Purification kit, Qiagen GmbH, Germany) after visualization in a 6% polyacrylamide gel. The membranes were pre-hybridized in 5 ×

SSPE, 5 × Denhardt's, 1% SDS, 5% dextran, 50% formamide, and ssDNA (200 µg/ml) for two hours at 42°C. The hybridization was carried out overnight at 42°C in the same solution after addition of the probes (100 ng). Membranes were rinsed once in 2 × SSC, 0.1% SDS for 15 min at room temperature and then washed three times in 0.1 × SSC, 0.1% SDS, first at room temperature for 15 min, and twice at 65°C for 30 and 15 min. Membranes were exposed overnight on a phosphorscreen and an autoradiography was obtained using a Storm 860 phosphorimager (Amersham Pharmacia Biotech, Piscataway, NJ). Densitometric analysis was carried out using Image-



**Figure 3**  
Expression of GATA-4 in neoplastic human adrenal tissues.

Quant 2.1 software (Amersham Pharmacia Biotech, Piscataway, NJ). Two normalizations were performed: 1 – densitometry data from tumor samples were normalized using the optical density from respective positive control, in order to correct any variation in transferring efficiency and probe activity. 2 – optical densities of GATA-4, GATA-6 and LHR were normalized for each tumor sample with corresponding cyclophilin densitometry data in order to obtain the relative expression levels. Intra and inter-assay coefficients of variation for GATA-4, GATA-6, and LHR are, respectively, 4.14% and 13.23%, 12.63% and 12.24%, 7.73% and 12.16%.

**Statistical analysis**

Relative expression of GATA-6, GATA-4 and LHR in non-metastasizing and metastasizing groups was compared using Student t test. The ability of empirically defined cut-off values of GATA-4, 6 and LHR transcripts to discriminate between these two groups was verified using Chi-square test.

**Results**

**Expression of GATA-6 and GATA-4 in human adrenocortical neoplasms**

The ability of both normal and tumoral human adrenal cortices and also a human adrenocortical cell line – NCI-H295A [38,39] – to express GATA-6 and GATA-4 was assessed by PCR amplification of reversely transcribed total RNA. In order to enhance the detection sensitivity and to perform quantitative analysis of GATA-6 and GATA-4 accumulation, amplification products from a second RT-PCR were then vacuum-transferred to a membrane and subjected to dot-blot hybridization. Both GATA-6 and GATA-4 transcripts were detectable in normal adrenal and in NCI-H295A cells, with GATA-6 being more abundant than GATA-4 (Figure 1). Thus, both GATA transcripts are normally expressed in human adrenal and NCI-H295A cells. GATA-6 mRNA was detected in all 23 tumors, irrespective of their behavior (Figure 2). Sample 16 yielded no visible amplification product on agarose gel but was clearly positive for GATA-6 in dot-blot (Figure 2),

**Table 2: GATA-6, GATA-4, and LHR expression in human adrenocortical tumors**

Patient	Tumor Evolution	GATA-6 Expression	GATA-4 Expression	LHR Expression
1	NM	2.43	1.09	0.54
2	NM	3.39	0.60	1.60
3	NM	3.58	1.75	1.60
4	NM	3.45	0.59	1.07
5	NM	3.21	0.05	1.64
6	NM	2.98	0.91	0.69
7	NM	2.59	0	0.83
8	NM	3.19	0.04	1.17
9	NM	2.62	0.70	1.00
10	NM	3.02	0.52	0.54
11	NM	3.45	0.14	3.84
12	NM	2.44	0.62	0.78
13	NM	3.11	0	1.57
14	MR	3.24	0.32	0.41
15	MR	2.30	3.50	0.44
16	MR	1.74	0.01	0.35
17	MR	2.81	2.50	0.41
18	MR	2.14	3.37	1.06
19	MR	2.61	2.17	0.33
20	MR	2.71	2.96	0.50
21	MR	2.83	0.12	0.48
22 <sup>a</sup>	MR	3.23	0.58	1.76
22 <sup>b</sup>	MR	2.07	2.23	1.83

Patients are identified as in Table 1. Relative expression levels were calculated normalizing optical densities of GATA-6, GATA-4 and LHR autoradiograms with corresponding cyclophilin densitometric data as described in materials and methods. Samples 22<sup>a</sup> and 22<sup>b</sup> are tumor specimens removed from left (22<sup>a</sup>) and right (22<sup>b</sup>) adrenal of the same patient (patient 22, Table 1) **NM**: non-metastasizing, **MR**: metastasizing/recurring

illustrating the higher detection sensitivity of this method. Amplification product corresponding to GATA-4 cDNA was detected in 11 out of 13 NM tumors and in 9 of the 10 MR tumors (Figure 3). Although a faint band, with the expected size for GATA-4, was visible in the lane corresponding to sample 16 (Figure 3), no signal was detected on dot-blot. Given the higher sensitivity and specificity of dot-blot, this sample was considered negative and the band regarded as an artifact. Densitometric analysis of the autoradiogram provided quantitative information regarding accumulation of GATA transcripts (Table 2). Non-metastasizing tumors expressed more GATA-6 than metastasizing ones (respectively,  $3.035 \pm 0.111$  and  $2.568 \pm 0.138$ ,  $p = 0.032$ ) while an opposite pattern was observed for GATA-4 ( $0.539 \pm 0.143$  and  $1.776 \pm 0.384$ ,  $p = 0.003$ ).

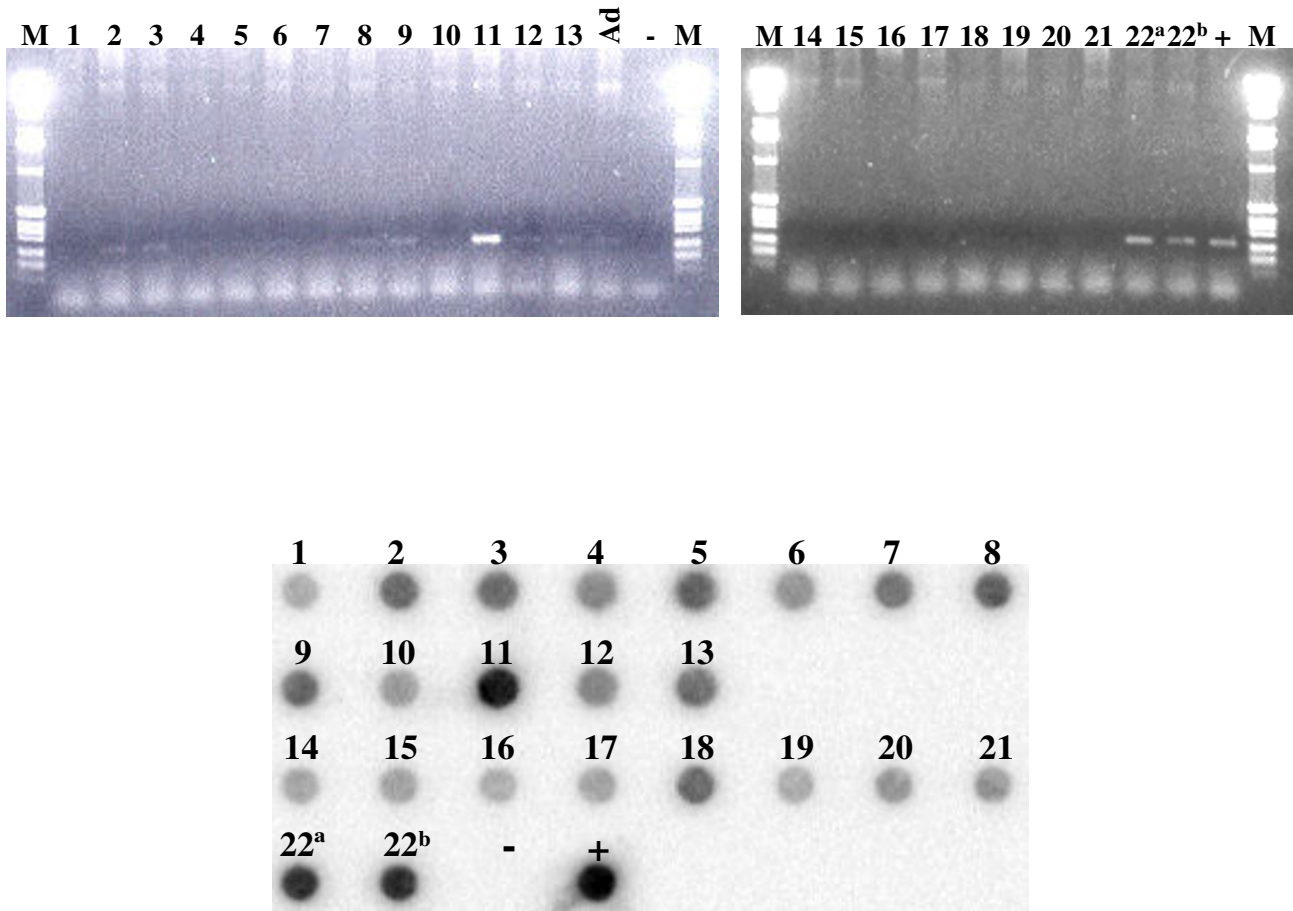
#### Expression of LH/hCG receptor in human adrenocortical neoplasms

Gonadotropins have been shown to up-regulate GATA-4 in gonadal tumor cell lines [30,32]. In mouse Leydig cell lines, GATA-4 mRNA accumulation is stimulated by hCG [30]. Since LH/hCG receptor has been detected in both normal human and tumoral mouse adrenal cortices [40,41], we speculated that LHR expression might be cor-

related with the accumulation of GATA-4 in human adrenal cortex. For this reason, we investigated the expression pattern of LH receptor in our adrenocortical specimens. Amplification products of LHR transcript were observed in all tumors, and also in normal adrenal cortex and NCI cells (Figures 1 and 4). Samples displaying more abundant LHR transcripts were, in their majority, NM tumors. Nevertheless, no statistically significant difference in LHR mRNA accumulation was found ( $1.298 \pm 0.24$  and  $0.757 \pm 0.162$ , respectively for NM and MR groups,  $p = 0.209$ ) in this group compared to the MR one. Samples expressing more abundant GATA-4 generally expressed smaller amounts of LHR (samples 15, 17, 19, 20, Figures 3, 4 and 6). Conversely, samples expressing smaller amounts of GATA-4 usually expressed LHR more intensely (samples 2, 5, 7, 8, 11, 13, Figures 3, 4 and 6).

#### Correlation between tumor behavior and expression of GATA-4, 6 and LHR

Since the relative expression of GATA transcripts in the NM group is different from those in the MR group (Table 2) we examined whether a cut-off value for the expression of such mRNAs could be used as a predictor of tumor behavior. In our cohort, an empirically established expression level of GATA-4 above 1.77 is correlated with poor



**Figure 4**  
 Expression of LHR in neoplastic human adrenal tissues. Expected size for LHR amplification product is 234 bp.

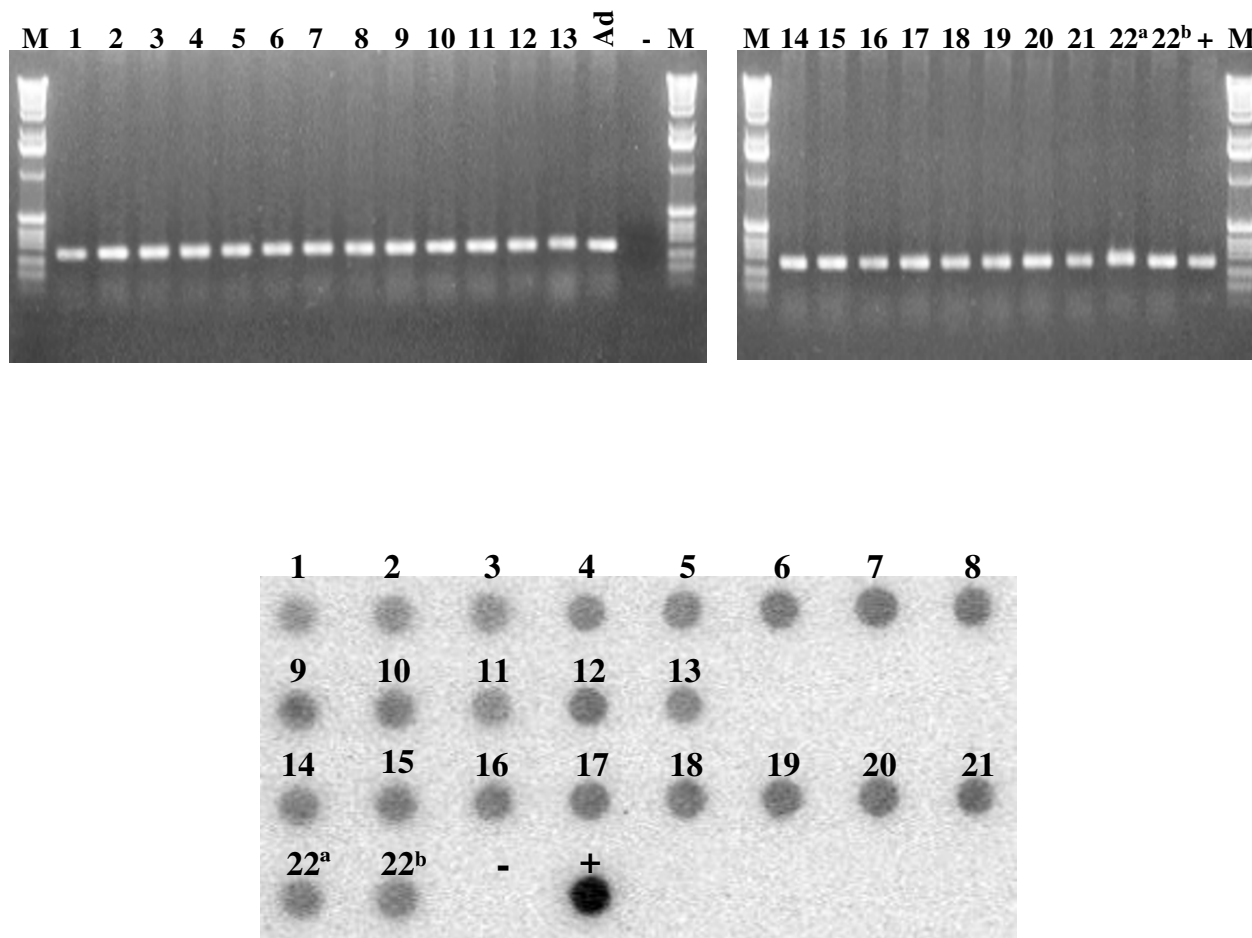
prognosis ( $p = 0.006$ ) with a relative risk of 4.25 (95% CI: 1.508 – 4.25). Such a cut-off value, however, would lead to a false negative diagnosis of 4 out of 10 MR subjects (patients 14, 16, 21, and 22a). In contrast, no cut-off allowing a clear discrimination between favorable and unfavorable outcome could be determined for GATA-6.

We also verified whether LHR expression could serve as a predictor for tumor outcome. Although NM and MR groups showed no differences regarding the relative expression of LHR, expression below an empirical threshold of 0.53 was correlated with aggressive behavior ( $p = 0.002$ ) with a relative risk of 5.333 (95% CI: 1.815 – 5.333). Combination of both GATA-4 and LHR did not improve any further the ability to discriminate tumoral behavior and clinical fate.

**Discussion**

In this report, carried out on 22 patients, we demonstrated that expression of GATA-4 in human adrenal cortex is not confined to malignant tumors, but also occurs in non-metastasizing tumors. GATA-6 transcripts were detected in all tumor samples, regardless of their biological behavior. We believe that the obtained RT-PCR products truly reflect the actual expression of GATA factors in those tissue samples and are not a result of amplification of GATA transcripts expressed in the stroma and capillaries surrounding the neoplastic adrenocortical cells. First, we were able to detect both GATA-6 and 4 transcripts in cultured human adrenal NCI-H295A cells (Figure 1), demonstrating that expression of these two members of the GATA family actually occurs in some cell lines of human adrenal cortex. Second, there are a number of unequivo-





**Figure 5**  
 Expression of cyclophilin in neoplastic human adrenal tissues. Integrity of total RNA used in RT-PCR experiments was confirmed by the presence of a 259 bp cyclophilin cDNA fragment in all tissue samples.

cally GATA-4 negative tissue specimens among those we have analyzed in this study (Figure 3), suggesting that the contribution of GATA-4 from the endothelial cells and surrounding tumoral stroma on the RT-PCR signal was negligible.

The highest GATA-4 expression levels are found among the MR samples. This suggests that a high relative expression of GATA-4 might be correlated with a poor outcome. Nevertheless, GATA-4 expression is not confined solely to malignant neoplasms of human adrenal cortex since 11 out of 13 NM tumors were GATA-4 positive. Our results are not consistent with a previous study, based on a model of adrenal tumorigenesis using inhibin  $\alpha$  promoter/SV40 Tag transgenic mice, which revealed that GATA-4 was up-

regulated while GATA-6 was down-regulated in carcinomas [37]. It is not clear why there were such differences between our data and those observed in the inhibin  $\alpha$  promoter/SV40 Tag transgenic mice [37]. One possible explanation relies on the difference in sensitivity of the detection method. In this study, we used RT-PCR followed by dot-blot, which is more sensitive than ribonuclease protection assay for detection of gene expression. Alternatively, given the probable relationship between the hypersecreted gonadotropins and adrenocortical tumorigenesis on these transgenic mice [41-43], we speculated that the reciprocal pattern of GATA-6 and 4 expression reported for this mouse model might be the result of an increased secretion of LH following gonadectomy.

Tumors expressing higher levels of GATA-4 tended to exhibit lower levels of LHR, while the opposite has also occurred. It is worth noting that both tumor samples removed from patient 22, who presented an unexpectedly benign evolution (Table 1), expressed the highest levels of LHR amongst the whole MR group (Table 2). In spite of presenting recurrent pulmonary metastases, this patient is presently alive and his disease is under control after surgical excision of those metastatic lesions. This observation suggests a possible correlation between long-term survival and higher expression of LHR. It remains to be confirmed whether a higher relative expression of LHR is associated with a less aggressive disease course.

We conclude that, in opposition to experimental mouse adrenocortical tumors, expression of GATA-6 in human adrenal cortex is not affected by tumorigenesis, and persists in both metastasizing and non-metastasizing neoplasms. In this small series of patients GATA-4 expression tends to be more intense in metastasizing tumors. Further studies with larger cohorts are warranted before one can reach a more definitive conclusion regarding the prognostic value of expression level of LHR or GATA-4 in adrenocortical neoplasms.

### Competing interests

None declared.

### Authors' contributions

A.S.B. carried out the molecular analyses, participated in the design of the study, and in the writing of the manuscript. L.R.G. reviewed the clinical data, participated in the design of the study, in the collection of specimens and in the writing of the manuscript. R.M.M. participated in the molecular analyses, and reviewed the clinical data. B.B.M. coordinated the collection of samples and the follow-up of the enrolled patients and participated in the design of the study. C.J.L. conceived the study, participated in the molecular analyses, in the interpretation of data and in the writing of the manuscript.

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