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Lack of in vitro constitutive activity for four previously reported TSH receptor mutations identified in patients with nonautoimmune hyperthyroidism and hot thyroid carcinomas

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The authors have nothing to declare

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Abstract

Objective: Constitutively activating mutations of the TSHR are the major cause for nonautoimmune hyperthyroidism. Re-examination of constitutive activity previously determined in CHO cell lines recently demonstrated the caveats for the *in vitro* determination of constitutive TSHR activity, which leads to false positive conclusions regarding the molecular origin of hyperthyroidism or hot thyroid carcinomas.

Design: Mutations L677V and T620I identified in hot thyroid carcinomas were previously characterized in CHO and in 3T3-Vill cell lines, respectively, stably expressing the mutant without determination of TSHR expression. F666L identified in a patient with hot thyroid nodules, I691F in a family with nonautoimmune hyperthyroidism and F631I identified in a hot thyroid carcinoma were not characterized for their *in vitro* function. Therefore, we decided to (re)evaluate the *in vitro* function of these five TSHR variants by determination of the cell surface expression, intracellular cAMP and IP levels and performed additionally linear regression analyses to determine the basal activity independently from the mutant's cell surface expression in COS-7 and HEK_{GT} cells.

Results and Conclusions: Only one (F631I) of the five investigated TSHR variants displayed constitutive activity for G_{αs} signaling and showed correlation with the clinical phenotype. The previous false classification of T620I and L677V as CAMs is most likely related to the circumstance that both mutations were characterized in cell lines stably expressing the mutated receptor construct without assessing the respective receptor number per cell. Other molecular etiologies for the nonautoimmune hyperthyroidism and/or hot thyroid carcinomas in these 3 patients and one family should be elucidated.

Introduction

Activating somatic mutations of the TSHR have been found in hot thyroid nodules and hot thyroid carcinomas. Activating TSHR germline mutations occur in familial nonautoimmune hyperthyroidism and sporadic congenital hyperthyroidism ¹. Moreover, inactivating mutations have been reported in cases of resistance to thyrotropin (TSH) ². Constitutively activating mutations (CAMs) of the *TSHR* gene induce a ligand-independent state of activation, which results in a permanent activation of the G_s- and in some cases also the G_q-mediated signaling pathway ^{3,4}.

However, also TSHR variants have been described, which did not affect *in vitro* TSHR function such as D727E ^{5,6} and Pro52Thr ⁷. Recently, Gozu et al. ⁸ described a TSHR germline mutation (N372T) located in the extracellular domain. It's association with euthyroidism in 13 family members was in line with it's lack of constitutive activity as demonstrated by linear regression analysis of constitutive activity as a function of TSHR expression (LRA). Moreover, Mueller et al. ⁹ demonstrated the limitations of the current methods to precisely determine if mutants with only a slight increase of the basal activity are constitutively active or not. This study showed that the characterizations of TSHR mutations regarding their constitutive activity should always include the determination of the LRA and should consider the influence of the *in vitro* cell and/or vector system on the basal cAMP accumulation. Especially the determination of the constitutive activity of the R310C substitution in CHO cell lines demonstrated the pitfalls for the *in vitro* determination of constitutive TSHR activity ^{9,10}. For most (90%) TSHR mutations the constitutive activity was determined *in vitro* using the COS-7 cell system ¹¹. Moreover, these studies also demonstrated that comprehensive clinical characterization of these patients is an important adjunct to classify the activity of these mutations.

The updating of the TSHR mutation database (<http://innere.uniklinikum-leipzig.de/tsh>) led to the identification of three *in vivo* TSHR variants with no functional *in vitro* characterization. Variant F631I has been identified in a patient with toxic metastasizing follicular carcinoma associated with a second TSHR mutation D633Y, which has previously been characterized in

COS-7 cells as constitutively active^{12;13}. TSHR mutation F666L has been found in a hot nodule of a patient with toxic multinodular goiter and I691F was identified in a family with nonautoimmune hyperthyroidism and was only shown to segregate with the phenotype of nonautoimmune hyperthyroidism.^{14;15} Furthermore, T620I and L677V have been identified in patients with a hot follicular carcinoma (T620I) and a hot Hürthle cell thyroid carcinoma (L677V) and were previously characterized in CHO and in 3T3-Vill cell lines, respectively, stably expressing the mutant without determination of TSHR cell surface expression^{16;17}.

Therefore, F631I, F666L and I691F were functionally characterized by determination of cell surface expression, basal and TSH-mediated cAMP production, basal and TSH-mediated IP accumulation and LRAs to evaluate their role in nonautoimmune hyperthyroidism and hot thyroid carcinomas. Furthermore, we re-examined the previously published constitutively activating mutations T620I and L677V in the COS-7 and HEK_{GT} cell system, respectively. Only F631I displayed constitutive activity for the cAMP pathway and seems to correlate with the clinical phenotype. The other four investigated mutations showed no constitutive activity for G_{αs} or G_{αq} in COS-7 cells, which was confirmed by LRA. Based on the lack of constitutive activity for T620I, L666F, L677V and I691F in COS-7 and HEK_{GT} cells other molecular etiologies for nonautoimmune hyperthyroidism or the hot thyroid carcinomas in these patients should be elucidated.

Material and Methods

Site-directed mutagenesis - TSHR mutants were constructed as previously described ¹⁸.

Cell culture and transient expression of mutant TSHRs - COS-7 cells were grown in high glucose Dulbecco's modified Eagle's medium (DMEM; PAA Laboratories, Pasching, Austria) supplemented with 10 % FCS, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco Life technologies, Paisley, UK) at 37 °C in a humidified 5% CO₂ incubator. Grip Tite™ 293 MSR cells (HEK_{GT} cells; Invitrogen Group, Paisley, UK) were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FCS, 1% nonessential amino acids (PAA Laboratories, Pasching, Austria), 100 U/ml penicillin, 100 µg streptomycin and 1% geneticin (Gibco Life technologies, Paisley, UK) at 37 °C in a humidified 5% CO₂ incubator. Cells were transiently transfected in 12-well plates (1 x 10⁵ cells per well for COS-7 and 1.4 x 10⁵ cells per well using HEK_{GT}) or 24-well plates (0.5 x 10⁵ cells per well for COS-7 and 0.7 x 10⁵ cells per well using HEK_{GT}) with 1 µg and 0.5 µg DNA per well, respectively, using the GeneJammer® Transfection Reagent (Stratagene, Amsterdam, NL).

FACS analyses - The TSH receptor cell surface expression level was quantified as described by Mueller et al. ⁹.

cAMP accumulation assay - Determination of intracellular cAMP levels were performed as described by Mueller et al. ⁹.

Linear regression analysis of constitutive activity as a function of TSHR expression (slopes) - The constitutive activity is expressed as basal cAMP formation as a function of receptor expression determined by FACS. COS-7 and HEK_{GT} cells were transiently transfected in 24-well plates with increasing concentrations of wt or mutant TSHR plasmid DNA (50; 100; 200; 300; 400 and 500 ng per well). To transfect a constant DNA amount of 500 ng empty vector plasmid DNA was added to the mutant or wt DNA. For determination of cell surface

expression see “FACS analyses”. Basal cAMP formation as a function of receptor expression was analyzed using the linear regression module of GraphPad Prism 4 for Windows.

Activation of inositolphosphate formation - Determination of inositolphosphate formation were performed as described by Jaeschke et al.¹⁹.

Statistics - Statistical analysis was carried out using the Mann-Whitney nonparametric *t* test using GraphPad Prism 4 for Windows.

Results

To evaluate the impact of the TSHR mutations with the patients clinical phenotype all mutations were functionally tested regarding cell surface expression, basal and TSH-mediated intracellular cAMP accumulation and basal and TSH-mediated production of inositolphosphates (IP). Furthermore, we performed linear regression analyses (LRA) to examine the basal activity independently from the mutant's cell surface expression. Several methods to measure the basal TSHR G_s activity *in vitro*, e.g. direct measurement of the cAMP concentration, a luciferase assay using the cAMP response element (CRE) and measurement of the adenylate cyclase activity have been used in recent studies²⁰. Currently the best method to measure ligand independent activation of the TSHR by mutations is the slope method. This approach allows experimentally the determination of the basal activity independently from the receptors cell surface expression^{9;20}.

The cell surface expression of the four investigated TSHR constructs was between 40 – 103% (wt TSHR = 100%) in COS-7 cells (Table 1) and in HEK_{GT} cells the TSHR mutations were expressed similarly (Table 2). Mutations T620I and F666L showed nearly the same expression as the wt TSHR. I691F was expressed to 88% in COS-7 cells and to 94% in HEK_{GT} cells. The lowest cell surface expression levels were determined for mutation L677V and F631I (Table 1 and 2).

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3 Interestingly, in both characterized cell lines four of the five characterized mutations showed
4 no increase in basal cAMP production. The relative fold increases were even slightly below
5 the basal activity measured for the wt TSHR (set = 1). This accounts for mutations T620I
6 (COS-7: 0.7; HEK_{GT}: 0.6), F666L (COS-7: 0.6; HEK_{GT}: 0.8) and I691F (COS-7: 0.8; HEK_{GT}:
7 0.9). The basal cAMP production for L677V was the same as determined for the wt TSHR in
8 COS-7 and was confirmed by the same basal activity in HEK_{GT} cells (Table 1 and 2). Only
9 variant F631I revealed constitutive activity in terms of an elevated basal cAMP level with a
10 relative fold change to 3.7 (COS-7) and 4.8 (HEK_{GT}) compared with the wt TSHR. TSH-
11 mediated signaling of T620I and F666L was unaltered whereas F631I, L677V and I691F
12 revealed a slight but significant decrease in cAMP production (Table 1). However, after TSH
13 administration in HEK_{GT} cells no significant changes in cAMP production were measured for
14 all investigated constructs when compared with the wt TSHR. (Table 2). The experimental
15 verification of the receptors constitutive activity by LRA in both cell systemy confirmed the
16 observation that mutations T620I, F666L, L677V and I691F were not constitutively active.
17 The slopes of the LRA experiment ranged from 0.4 to 1.4 in COS-7 cells (wt TSHR = 1) and
18 were in between 0.4 and 1.8 in HEK_{GT} cells. In contrast, F631I was confirmed to be a
19 constitutively active mutant by LRA with a slope of 8.6 and 19.0, respectively (Table 1 and
20 2).
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22 The basal IP levels of the five receptor constructs showed no discrepancies in comparison
23 with the wt TSHR. A reduction of intracellular IP production after TSH stimulation was
24 observed for F631I, T620I, F666L and L677V, whereas I691F IP accumulation after TSH
25 administration was comparable with the wt TSHR. (Table 1 and 2).
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Discussion

In this study we functionally characterized three TSHR variants (F631I, F666L and I691F) previously reported in patients with hyperthyroidism and/or hot thyroid carcinomas for the first time. These TSHR variants were previously identified in patients with toxic metastasizing follicular carcinoma and with a toxic multinodular goiter, respectively, and in a family with nonautoimmune hyperthyroidism^{12;14;15}. With an LRA of 8.6 the mutation F631I is clearly constitutively active (Table 1, Figure 1). However, F666L and I691F did not display constitutive activity in terms of increased basal activity for the $G_{\alpha s}$ - and the $G_{\alpha q}$ -pathway. Moreover, F666L is not able to activate $G_{\alpha q}$ after TSH administration, whereas I691F displayed a similar phenotype like the wt after TSH stimulation for $G_{\alpha q}$. These findings support the conclusion that F666L and I691F are not constitutively activating. The constitutively activating mutation F631I has been identified in a patient with thyroid follicular cancer and was associated with two toxic thyroid nodules. In this patient each nodule contained a different TSHR mutation namely D633Y and F631I¹². Further genetic alterations *e.g.* of the *RAS* gene or *PAX8-PPAR γ* rearrangements, which might contribute to the phenotype of follicular carcinoma, were excluded in this patient. Variant D633Y was previously identified by Porcellini and co-workers in a patient with nonautoimmune hyperthyroidism²¹ and a comprehensive functional characterization confirmed that this amino acid substitution was the molecular origin of hyperthyroidism¹³. With the functional data of the newly characterized F631I variant we confirmed that the patient with thyroid follicular cancer harbored two different CAMs.

The *in vitro* functionally neutral variant F666L, which was previously identified in a hot nodule of a patient with toxic multinodular goiter could be associated with a further constitutively activating somatic TSHR mutation like in the patient reported by Gozu et al.²². The previously reported *in vitro* neutral D727E variant has been identified in 12 of 56 (21.4%) hot nodules in the nodular tissue and also in DNA extracted from the lymphocytes²². In one patient it was found homozygous and accompanied by the constitutively activating TSHR mutation D633Y and in 11 patients the polymorphism was heterozygous. Alternatively

and more likely it is associated with a further currently unknown molecular cause for the hot nodule, which remains to be identified in about 30 - 40% of the hot nodules without somatic TSHR mutations^{22,23}.

The *in vitro* functionally neutral TSHR variant I691F identified in a family with nonautoimmune hyperthyroidism and shown to segregate with the phenotype of nonautoimmune hyperthyroidism is located in the intracellular cytoplasmic part of the TSHR. A further *in vitro* functionally neutral variant is known in this region, D727E, which shows wt like expression levels and signaling capacities^{5,6}. Also for the TSHR related LHCGR and FSHR no mutations leading to an elevated basal cAMP level in the homologous region have been reported. The occurrence of a neutral TSHR germline variant was recently shown in a Turkish family. All 13 family members carried the neutral substitution N372T and were euthyroid. Only one family member showed hyperthyroidism due to a second somatic mutation at position 281 (S to N), which turned out to be the molecular cause of hyperthyroidism in this single family member. Therefore, the occurrence of a TSHR germline mutation in a family (even if it segregates with hyperthyroidism) is not necessarily an argument for its constitutive activity.

The variants T620I and L677V detected as somatic mutations in a hot follicular carcinoma (T620I) and in a hot Hürthle cell thyroid carcinoma (L677V) and previously characterized in CHO and in 3T3-Vill cell lines, respectively, are also not constitutively activating for the G_αs-mediated pathway. F666L even showed a reduction for the TSH-mediated G_αq activation. The previous false classification of T620I and L677V as CAMs is most likely related to the circumstance that both mutations were characterized in cell lines stably expressing the mutated receptor construct without assessing the respective receptor numbers per cell^{16,17}.

The basal activity of GPCRs tightly correlates with the number of receptors expressed on the cell surface. It is unlikely that the receptor numbers of the cell line harboring the wt TSHR was the same as the cell line expressing the mutation. A comparison of the receptors basal activity in relation to the wild type is impossible without the determination of the receptor cell surface expression. Therefore, the best experimental approach to characterize the basal

activity independently from the receptors cell surface expression is the determination of the LRA ⁹. The resulting slopes are to date the best parameter to determine if a receptor is constitutively active. This was previously shown for the TSHR mutation R310C, which was also initially characterized in CHO cells stably expressing the mutated receptor construct ¹⁰. However, LRA studies of this particular TSHR mutation after transient expression in COS-7 and HEK_{GT} cells revealed no constitutive activity ⁹.

In the patient with a follicular thyroid carcinoma besides the *in vitro* functionally neutral T620I TSHR variant another point mutation in the *Ki-RAS* gene (G12C) was identified ¹⁶. In follicular thyroid carcinomas *RAS* mutations occur in 40-50% and are considered to represent an early step in tumor formation ²⁴. Moreover, *PAX8/PPAR γ* rearrangements can be identified in follicular carcinomas. This was not investigated in this case report. No further candidate genes were screened in the hot Hürthle cell carcinoma with the *vitro* functionally neutral L677V TSHR variant ¹⁷. Therefore, the molecular cause for the hot thyroid carcinoma in this patient remains unknown.

Up to date, ten activating TSHR mutations have been reported in nine differentiated hot thyroid carcinomas (Table 3). Among these five follicular carcinomas with six different activating TSHR gene mutations: I486F ²⁵, T620I ¹⁶, F631I and D633Y ¹², T632A and T632I ²⁶ were reported. In addition, a D633H mutation was identified in a hot insular thyroid carcinoma ²⁷, a M453T mutation was detected in a 11-year-old girl with a hyperfunctioning thyroid papillary carcinoma ²⁸, a L512R mutation was found in an autonomously functioning papillary carcinoma ²⁹ and L677V was identified in a hot Hürthle cell carcinoma ¹⁷. Except for the *vitro* functionally neutral T620I and V677L variants, which were previously characterized in cell lines stably expressing the receptor construct the other TSHR mutations found in hot thyroid carcinomas were done by transient expression in COS-7 cells with the results summarized in Table 2. Therefore, only the hot thyroid carcinomas with the TSHR mutations M453T, I486F, L512R, F631I, T632A, T632I, D633H and D633Y are associated with constitutively activating TSHR mutations.

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For Peer Review

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Table 1. Functional characterization of the TSHR constructs in COS-7 cells

Construct	Localization	Cell surface expression	cAMP accumulation			IP accumulation $IPs(\%[IPs+PI])$	
			basal	100mU/ml TSH	LRA (slope)	basal	100mU/ml TSH
wt TSHR	-	100	1	15.6 ± 0.5	1	3.8 ± 0.4	30.8 ± 6.2
pcDNA	-	3 ± 1	0.2 ± 0.1	0.3 ± 0.1	-	4.1 ± 0.9	4.2 ± 1.1
T620I	TMH6	103 ± 2	0.7 ± 0.3	17.1 ± 0.9	0.4 ± 0.1	4.0 ± 0.2	19.4 ± 0.5
F631I	TMH6	40 ± 2*	3.7 ± 0.5 **	12.0 ± 0.9**	8.6 ± 0.7*	4.2 ± 0.4	6.4 ± 0.4
F666L	TMH7	98 ± 9	0.6 ± 0.1	14.7 ± 3.7	0.8 ± 0.1	2.9 ± 0.7	9.2 ± 1.3
L677V	TMH7	66 ± 5*	1.0 ± 0.2	12.1 ± 1.1**	1.4 ± 0.6	3.7 ± 0.4	7.6 ± 1.3
I691F	C-term.	88 ± 6	0.8 ± 0.1	11.2 ± 0.6**	0.7 ± 0.1	4.2 ± 0.4	28.7 ± 0.8

COS-7 cells were transfected with wt TSHR or various mutant TSHRs. The vector pcDNA3.1(-)/hygromycin was used as a control. The TSHR is characterized by an elevated cAMP level compared to the control vector alone³⁰. Therefore, cAMP accumulation is expressed relative to wt TSHR basal level. TSH-mediated levels of cAMP and IP accumulation were determined after treatment of cells with 100mU/ml TSH. Expression of wt and mutant TSHRs were quantified on a FACS flow cytometer. Data are given as mean ± standard deviation (SD) of at least two independent experiments (n = 2), each carried out in duplicate. Constitutive activity by linear regression analyses was determined for G_αs- and not for G_αq-signaling due to the fact that the wt TSHR do not show elevated G_αq-levels. (TMH = transmembrane helix; C-term. = C-terminus); ***p<0.001, **p=0.001 – 0.01, *p=0.01 – 0.05

Table 2. Functional characterization of the TSHR constructs in HEK_{GT} cells

Construct	Localization	Cell surface expression	cAMP accumulation			IP accumulation <i>IPs</i> (% <i>IPs</i> + <i>PII</i>)	
			<i>basal</i>	100mU/ml TSH	<i>LRA (slope)</i>	<i>basal</i>	100mU/ml TSH
wt TSHR	-	100	1	40.0 ± 1.9	1	0.6 ± 0.1	10.7 ± 1.8
pcDNA	-	1 ± 0	0.3 ± 0.1	0.3 ± 0.1	-	0.4 ± 0.0	0.6 ± 0.1
T620I	TMH6	97 ± 5	0.6 ± 0.3	45.1 ± 5.0	0.4 ± 0.1	0.6 ± 0.1	6.0 ± 0.4*
F631I	TMH6	46 ± 3**	4.8 ± 0.5*	37.5 ± 3.8	19.0 ± 4.0**	0.6 ± 0.1	1.1 ± 0.0*
F666L	TMH7	106 ± 8	0.8 ± 0.1	41.2 ± 4.1	0.8 ± 0.2	0.5 ± 0.0	5.5 ± 0.6*
L677V	TMH7	77 ± 4**	1.0 ± 0.2	39.4 ± 7.0	1.8 ± 0.3	0.6 ± 0.0	2.7 ± 0.3*
I691F	C-term.	94 ± 2	0.9 ± 0.1	45.9 ± 3.9	1.6 ± 0.2	0.6 ± 0.0	11.8 ± 0.8

HEK_{GT} cells were transfected with wt TSHR or various mutant TSHRs. The vector pcDNA3.1(-)/hygromycin was used as a control. The TSHR is characterized by an elevated cAMP level compared to the control vector alone³⁰. Therefore, cAMP accumulation is expressed relative to wt TSHR basal level. TSH-mediated levels of cAMP and IP accumulation were determined after treatment of cells with 100mU/ml TSH. Expression of wt and mutant TSHRs were quantified on a FACS flow cytometer. Data are given as mean ± standard deviation (SD) of at least two independent experiments (n = 3), each carried out in duplicate. Constitutive activity by linear regression analyses was determined for G_αs- and not for G_αq-signaling due to the fact that the wt TSHR do not show elevated G_αq-levels. (TMH = transmembrane helix; C-term. = C-terminus) ; ***p<0.001, **p=0.001 – 0.01, *p=0.01 – 0.05

Table 3. All patients reported up to date with a differentiated hot thyroid carcinoma and a constitutively activating TSHR mutation.

Construct	Reference	Cell system	Cell surface expression	cAMP accumulation		
				<i>basal</i>	<i>100mU/ml TSH</i>	<i>LRA (slope)</i>
wt TSHR	-		100%	100%	100%	1
M453T	29	-	-	-	-	-
	32	COS-7	-	700	80	-
	19	COS-7	66	570	84	5.2
I486F	26	-	-	-	-	-
	33	COS-7	20	400	-	-
	34	COS-7	17	550	-	-
	35	COS-7	35	820	83	-
	36	COS-7	15	663	-	-
L512R	30	-	-	-	-	-
	37	COS-7	56	325	49	-
T620I	16	3T3 Vill	-	500	75	-
		COS-7	103	70	110	0.4
F631I	12	-	-	-	-	-
		COS-7	40	370	77	8.6
T632A	24	-	-	-	-	-
	33	COS-7	50	330	-	-
	38	COS-7	40	420	145	-
T632I	24	-	-	-	-	-
	13	COS-7	36	370	82	-
	39	COS-7	90	500	100	-
D633H	25	-	-	-	-	-
	36	COS-7	84	620	67	-
	40	COS-7	123	725	-	-
D633Y	12	-	-	-	-	-
	13	COS-7	60	405	88	-
L677V	17	CHO	-	343	-	-
		COS-7	66	100	78	1.4

Values with grey background were newly determined in this study demonstrating no constitutive activity for L677V and T620I. Only the mutations M453T, I486F, L512R, F631I, T632A, T632I, D633H and D633Y are constitutively activating. The data base sequence-structure-function analysis for glycoprotein hormone receptors was used¹¹.