

Gene expression analysis reveals evidence for inactivation of the TGF- β signaling cascade in autonomously functioning thyroid nodules

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Molecular events that lead to the development of autonomously functioning thyroid nodules (AFTNs) are somatic mutations of the thyrotropin receptor (TSHR) in approximately 60% of the nodules and less frequently, somatic mutations in the G_s α protein. However, AFTNs without known mutations indicate that other causes remain to be identified. Moreover, the impact of constitutively activating TSHR mutations on the signal transduction network of the thyroid epithelial cell is unknown. We therefore investigated gene expression in 15 AFTNs and their surrounding tissue using Affymetrix GeneChips. Most prominently, data analysis revealed a changed pattern of gene expression in the TGF- β signaling cascade and 25 differentially regulated genes in AFTNs, including thyroid peroxidase, type I iodothyronine deiodinase and sialyltransferase (SIAT) 1. Strikingly coexpression of SIAT 1 and TSHR in COS-7 cells increased TSH binding and cell surface expression of the TSHR. Moreover, differences in gene expression patterns for AFTNs with and without TSHR mutations indicate specific alterations of signal transduction in AFTNs without TSHR mutations. These results suggest that AFTNs with TSHR mutations harbor further mechanisms of forward stimulation. Furthermore, they give important leads to elucidate the molecular etiology of AFTNs without TSHR mutations.

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Introduction

In spite of large variations of the time spans (months to 17 years) for transitions of autonomously functioning thyroid nodules (AFTNs) from subclinical to overt hyperthyroidism (Sandrock *et al.*, 1993; Paschke and Ludgate, 1997), there is no genotype–phenotype corre-

lation for constitutively activating TSHR mutations (Führer *et al.*, 1999), which can be found in 60% of all cases (Parma *et al.*, 1993; Arturi *et al.*, 1998). It is therefore very likely that unknown signaling events, apart from counter-regulatory mechanisms like arrestins (Voigt *et al.*, 2000) or GRKs, contribute to the etiology of AFTNs and the clinical phenotype. Such events could be coupling of the TSHR to further downstream cascades in addition to cAMP and IP (Park *et al.*, 2000) or a shift of the coupling specificity (Biebermann *et al.*, 1998) or other TSHR-independent alterations in the signal transduction of the thyroid. Data about altered signal transduction or changed expression of oncogenes, tumor suppressors, or signaling molecules in thyroid nodules are rare. However, differentially expressed genes in nodular versus surrounding thyroid tissue could present a molecular signature of the development of AFTNs and benign tumors in general. Moreover, alterations of expression of signal molecules in TSHR mutation negative AFTNs could help to identify their molecular cause.

A limited recent investigation of 588 genes by cDNA expression array showed changes that seem to reflect a dominance of a specific signaling pathway (e.g. cAMP signaling) (Eszlinger *et al.*, 2001b). To gain a higher resolution, we here compare gene expression for approximately 10 000 full-length genes between AFTNs and their corresponding normal surrounding tissue (ST) using the U95A Affymetrix GeneChip. Regulation of gene expression in AFTNs was most consistent for sialyltransferase (SIAT) 1, where upregulation is found in all AFTNs studied. To investigate the importance of this regulation, we show that the TSHR is very likely a substrate for this enzyme in COS-7 cells since coexpression increases TSH binding and TSHR expression. Furthermore, we found a changed pattern of gene expression in the transforming growth factor (TGF)- β signaling pathway between AFTNs and their corresponding STs, that further explains findings of an increased proliferation in the nodular tissue (Krohn *et al.*, 1999). The differences in gene expression patterns for AFTNs with and without TSHR mutations indicate specific alterations of signal transduction in AFTNs without TSHR mutations.

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Results

GeneChips

Statistical examination of preliminary results of five AFTNs and their STs revealed the necessity to analyse at least 13 AFTNs and STs to detect twofold differences between AFTNs and STs with statistical reliability ($\alpha=0.001$, $\beta=0.05$, $s=1.60$). Therefore, we generated gene expression profiles for 15 AFTNs and their corresponding STs using U95Av2 GeneChips. Furthermore, the hybridization of one AFTN was repeated twice. The first repetition was scanned on the same confocal scanning microscope as the other AFTNs and STs, whereas the second repetition was scanned on a second confocal scanning microscope. We found a high correlation between the three samples ($R^2=0.94$ and 0.91 , respectively).

Empirical filtering

Empirical filtering of the data sets of the 15 AFTNs and their STs revealed 10 up- and 10 downregulated genes (Tables 1 and 2). Collagen type IX is the gene with the strongest increase in its expression in AFTNs in comparison to the STs (signal log ratio of 3.4). Furthermore, we found a strong and consistent increase in the expression of sialyltransferase 1 (beta-galactoside alpha-2,6-sialyltransferase, SIAT 1) and in the expression of two thyroid-specific genes: type I iodothyronine deiodinase (DIO 1) and thyroid peroxidase (TPO). Apolipoprotein D (Apo D) and member 21 of the small inducible cytokine subfamily A show strongest regulation in the group of downregulated genes (signal log ratios -3.5 and -3.2 , respectively, Table 2).

Statistical analysis according to Westfall–Young

At a significance level of $P<0.01$, the Westfall–Young procedure detected 45 differentially expressed genes.

Here, we find an overlap of 17 differentially expressed genes between empirical filtering and statistical analysis, whereas at a significance level of $P<0.001$, the Westfall–Young procedure detected 13 differentially expressed genes with an overlap of eight genes to the empirical filtering (Tables 1 and 2). Statistical analysis of the expression data revealed one additional, upregulated gene ($P<0.001$, AFTN versus ST): an EST clone with a high similarity to metallothionein 1B (Table 1). In the group of downregulated genes, we detected four additional genes with a P -value <0.001 : myocilin, chordin-like, protocadherin 9, and alcohol dehydrogenase 2 (Table 2).

Hierarchical clustering

Hierarchical clustering was used to interpret the patterns of gene expression between AFTNs and their normal STs. The clustering software organizes gene expression data, so that the genes (rows) and tissue samples (columns) are arranged according to the degree of similarity in their pattern of gene expression.

For the expression level of 725 genes (trimmed data set 1, AFTNs versus STs), clustering divided all tissue samples (AFTN and ST) into two major clusters (Figure 1). Interestingly, 15 normal STs grouped with two AFTNs (AFTN 114 and AFTN 136) within cluster 1 and the remaining 13 AFTNs clustered tightly together within cluster 2. The clustering of AFTN 114 and 136 in the group of STs might be caused by a cystic degeneration of AFTN 114 as well as a focal thyroiditis with lymph follicles in its ST and extensive nodular alterations in the ST of AFTN 136, respectively. The group of upregulated genes shows an eight-gene-overlap with our empirical and statistical algorithms (e.g. SIAT 1, thyroid peroxidase and four metallothioneins). In the cluster of downregulated genes, we found six genes that overlap with empirical filtering and statistical analysis. Additionally,

Table 1 Upregulated genes according to empirical filtering (signal log ratio >0.585 and difference call is 'increased' in 13 of 15 AFTNs in comparison to their corresponding STs) and Westfall–Young procedure ($P\leq 0.001$)

ProbeSet	Name	Symbol	Mean of signal log ratios	s.e.m.	P-value	Annotation to biological processes
36455_at	Collagen type IX alpha 3	COL9A3	3.4	0.5	0.0006	
41352_at	Sialyltransferase 1	SIAT1	2.1	0.2	0.0004	Protein modification, humoral defense mechanism
31966_at	Type I 5 iodothyronine deiodinase	DIO1	2.0	0.2	0.0001	
35928_at	Thyroid peroxidase	TPO	1.7	0.2	0.0012	Thyroid hormone biosynthesis
39333_at	Collagen type IV alpha 1	COL4A1	1.3	0.1	0.0005	
926_at	Metallothionein 1G	MT1G	1.3	0.2	0.0012	
39594_f_at	Metallothionein 1H	MT1H	1.2	0.1	0.0011	
31623_f_at	metallothionein 1A functional	MT1A	1.1	0.1	0.0007	Heavy metal resistance, heavy metal response, heavy metal ion transport
31844_at	Homogentisate 1 2-dioxygenase	HGD	1.1	0.1	0.0289	
31622_f_at	Metallothionein 1F functional	MT1F	1.0	0.1	0.0054	Heavy metal response
609_f_at	ESTs, highly similar to metallothionein 1B	MT1B2	0.9	0.1	0.0010	

The ProbeSet-ID, the names of the detected genes and their gene symbols are shown. Furthermore, the mean of the signal log ratios determined by the Affymetrix software for the 15 investigated AFTNs \pm s.e.m. are given. The adjusted P -value for each gene is given, which was calculated according to the Westfall–Young procedure, which imbeds a t -test into a permutation procedure. Genes detected by empirical filtering are given in italics

Table 2 Downregulated genes according to empirical filtering (signal log ratio < -0.585 and difference call is 'decreased' in 13 of 15 AFTNs in comparison to their corresponding STs) and Westfall-Young procedure ($P \leq 0.001$)

ProbeSet	Name	Symbol	Mean of Signal log ratios	SEM	P-value	Annotation to biological processes
36681_at	Apolipoprotein D	APOD	-3.5	0.3	0.0001	Lipid metabolism
36503_at	Member 21 of small inducible cytokine subfamily A	SCYA21	-3.2	0.4	0.0004	Immune response, signal transduction, inflammatory response, chemotaxis, cell-cell signalling
40403_at	Myocilin	MYOC	-3.1	0.3	0.0001	
37630_at	Chordin-like	LOC 57803	-2.4	0.4	0.0004	
36939_at	Glycoprotein M6A	GPM6A	-2.3	0.3	0.0003	
37394_at	Complement component 7	C7	-2.2	0.3	0.0042	Immune response, response to pathogenic bacteria
39878_at	Protocadherin 9	PCDH9	-2.2	0.2	0.0001	
1736_at	Insulin-like growth factor binding protein 6	IGFBP6	-2.1	0.4	0.2673	Signal transduction, negative control of cell proliferation
35730_at	Alcohol dehydrogenase 2	ADH 2	-2.0	0.2	0.0007	Ethanol metabolism
32664_at	Ribonuclease A family 4	RNASE4	-1.9	0.3	0.032	
32250_at	H factor 1 complement	HF1	-1.8	0.3	0.008	Complement activation
342_at	Nucleotide pyrophosphatase	NPP	-1.7	0.2	0.0045	
32805_at	Hepatic dihydrodiol dehydrogenase	HD-DH	-1.4	0.2	0.0045	
39757_at	Syndecan 2	SDC2	-1.1	0.1	0.0044	Cell adhesion, developmental processes

See Table 1 footnote

this gene cluster contains three members of the complement system, IGFBP 5, apolipoprotein J (clusterin) and platelet-derived growth factor receptor alpha.

Clustering of AFTNs with or without a TSHR mutation using the expression levels of 714 genes (trimmed data set 2) separated all AFTNs into two major groups (Figure 2). Strikingly, five AFTNs without a TSHR mutation were separated into cluster 1 and the remaining 10 AFTNs with a detected TSHR mutation into cluster 2. This separation is based on a group of 67 upregulated genes in AFTNs with a TSHR mutation in comparison to those without a mutation in the TSHR (four subclusters, Figure 2) and a second group of 33 downregulated genes in AFTNs with a TSHR mutation (in four subclusters, Figure 2). Within the two groups of genes, a considerable number of molecules related to signal transduction are found (e.g. upregulated genes are: G protein beta 3, p21-activated kinase 1 and 2; downregulated genes are: RGS 4, protein kinase C beta 1, Janus kinase 1, Rho 6, RGS 6, G protein-coupled receptor kinase (GRK) 2, interleukin 10 receptor beta).

GenMAPP

GenMAPP software was used for viewing and analysing our GeneChip-data on microarray pathway profiles (MAPPs). In the MAPP showing the TGF- β signaling pathway significant changes in the gene expression of associated genes could be identified (Figure 3). We found a significantly decreased expression of the TGF- β receptor type III (betaglycan), Smad 1, 3 and 4, ERK1 and P300. In contrast, the expression of inhibin, endoglin, Smad 6 and 7, and PAI-1 is significantly increased in AFTNs in comparison to their corresponding STs.

Real-time RT-PCR

The GeneChip expression data of eight differentially expressed genes (SIAT 1, DIO 1, TPO, IGFBP 6, metallothionein 1G, Apo D, PAI-1 and Smad3) were verified by real-time RT-PCR. These experiments show a high concordance between the GeneChip data and the real-time RT-PCR (Figure 4) even in cases with a lower, but significant differential expression (e.g. Smad3).

Dot blot

The expression of the TGF- β receptor type III in AFTNs and their ST, determined by GeneChip hybridization and by real-time RT-PCR, was also determined at the protein level by dot blot analysis. These experiments also show a significantly decreased expression of the TGF- β receptor type III in AFTNs in comparison to their ST (Figure 5).

FACS analysis, ELISA, and radioligand binding assay

The cell surface expression of the wild-type (wt) TSHR cotransfected with the SIAT 1 was $148 \pm 5.1\%$ in comparison to the wt TSHR, which was cotransfected with an empty pcDNA-vector. The investigation of TSHR cell surface expression by ELISA confirmed the FACS data. The ELISA showed an increase of TSHR cell surface expression to $144 \pm 19\%$. Moreover, cotransfection of SIAT 1 markedly increased the binding of labeled TSH by the receptor ($B_{\max} = 195\%$ in comparison to the wt TSHR). To determine whether the increased TSH binding after SIAT 1 cotransfection can be attributed to the increased cell surface expression of the TSHR, we reduced the cell surface expression of the TSHR in SIAT 1 cotransfection experiments by additional cotransfection

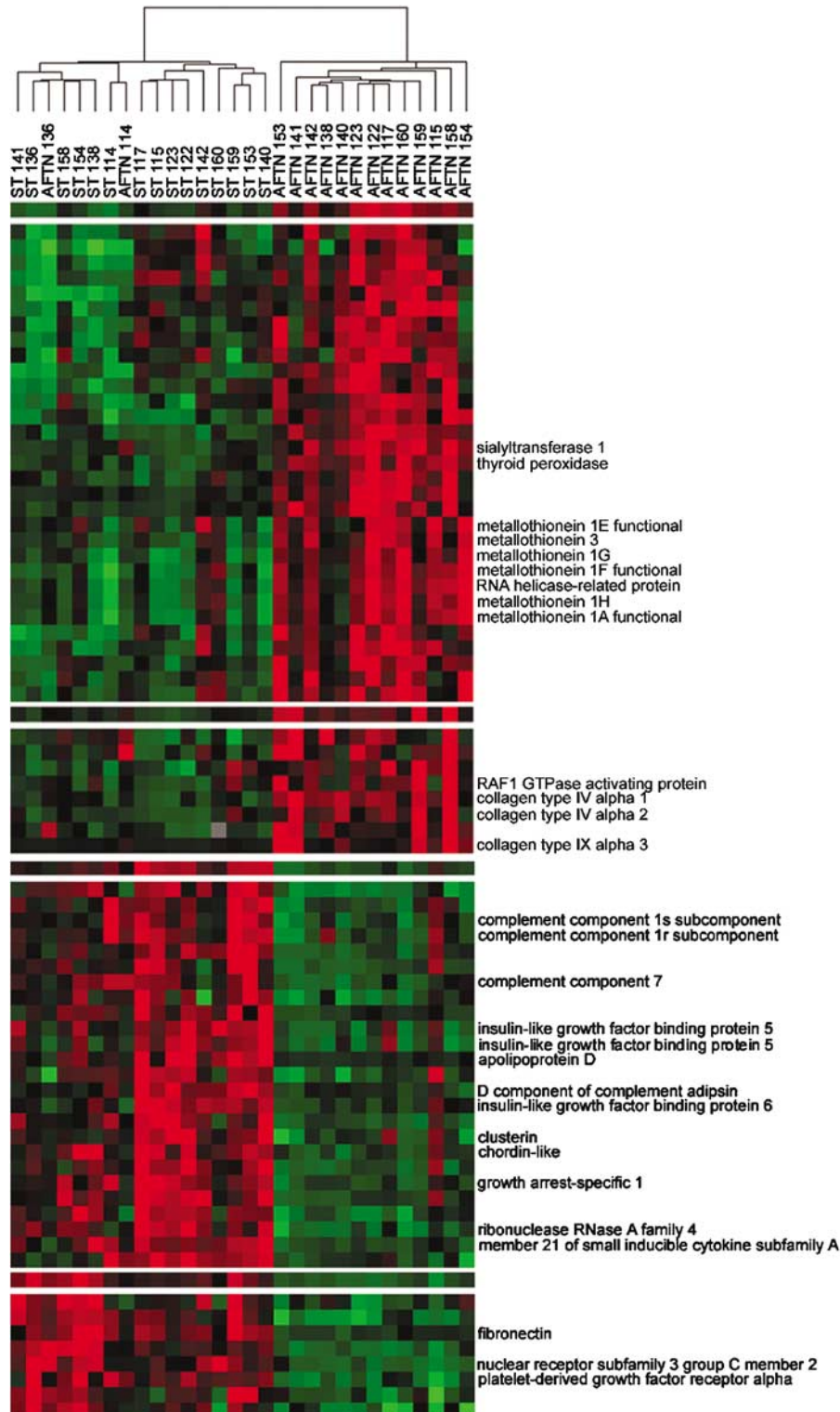


Figure 1 Comparison of the signal values of AFTNs and their corresponding normal STs by hierarchical clustering using the Cluster- and Treeview-software. Red color indicates upregulated genes, whereas green color indicates downregulated genes

of the human V2 receptor (V2R) as previously described (Neumann *et al.*, 2001). Thus, the TSHR cell surface expression was reduced below 100% (FACS: $76 \pm 6\%$, ELISA: $85 \pm 10\%$) of the reference experiment

(transfection of wt TSHR with an empty pcDNA-vector). Concurrent with the reduced cell surface expression of the TSHR, the binding of labeled TSH by the TSH receptor decreased ($B_{\max} = 81 \pm 5\%$).

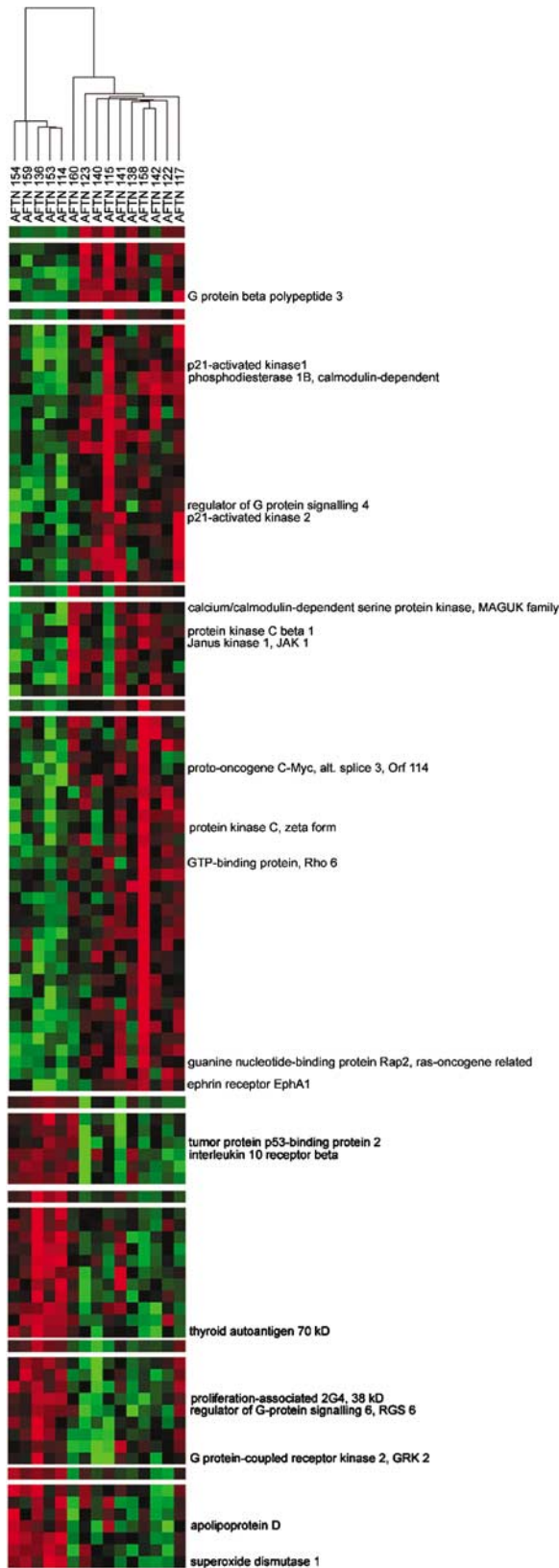


Figure 2 Comparison of the signal log ratios of AFTNs with TSHR mutations and AFTNs without TSHR mutations by hierarchical clustering using the Cluster- and Treeview-software. Red color indicates upregulated genes, whereas green color indicates downregulated genes

Discussion

We found a group of about 20–40 genes that are differentially expressed in nodular compared to normal thyroid tissue, which implies a role in the molecular mechanism of AFTNs. However, we now only start to understand the functional consequences for thyroid physiology when these genes are up- or downregulated. Most prominently, data analysis revealed a changed pattern of gene expression in the TGF- β signaling cascade that suggests a suppression of the TGF- β response. Moreover, we found 25 differentially regulated genes in AFTNs, including thyroid peroxidase, type I iodothyronine deiodinase, and SIAT 1. With the focus on SIAT 1, which codes an enzyme that transfers sialic acid residues to glycosylated proteins, we were able to elucidate transcriptional regulation that could be the basis of a new regulatory mechanism of TSH stimulation.

Clustering of gene expression for AFTNs with and without TSHR mutations revealed another set of differentially regulated genes with a clear dominance of signal transduction-related molecules. This finding supports the hypothesis that AFTNs without a TSHR or $G_s\alpha$ mutation are very likely a different entity with a different stimulus for nodular development and not just an unlikely detection failure for TSHR or $G_s\alpha$ mutations (Trulzsch *et al.*, 1999, 2001).

Data analysis

We used three different methods to detect differentially expressed genes. The strength of our empirical filtering was the simplicity of the approach that can be performed using common spreadsheet software for calculation (e.g. Microsoft-Excel). We were especially interested to see how our simple filtering algorithm not based on statistical tests or sophisticated calculations would compare to other algorithms. In general, we found a high overlap for all three applied methods. Although our empirical analysis only detected a small number of genes their differential expression between AFTNs and STs is solid, highly consistent and reproducible with quantitative RT-PCR. The Westfall-Young procedure gives a long list of genes ordered according to the statistical strength of the differences. Here, we also find genes that show weaker differential expression that is, nevertheless, statistically significant (Tables 1 and 2). In contrast, clustering defines the largest number of genes often not detected with the two other methods because the differential expression of these genes is weaker. However, clustering reveals a group of genes (e.g. signal transduction proteins) that share a characteristic pattern of expression and suggest a coordinated transcriptional regulation.

The expression data for six genes were verified by real-time RT-PCR. Only for TPO, the extent of differential gene expression is significantly higher for GeneChip data versus real-time RT-PCR. Therefore, we are confident that our results from GeneChips are highly reliable.

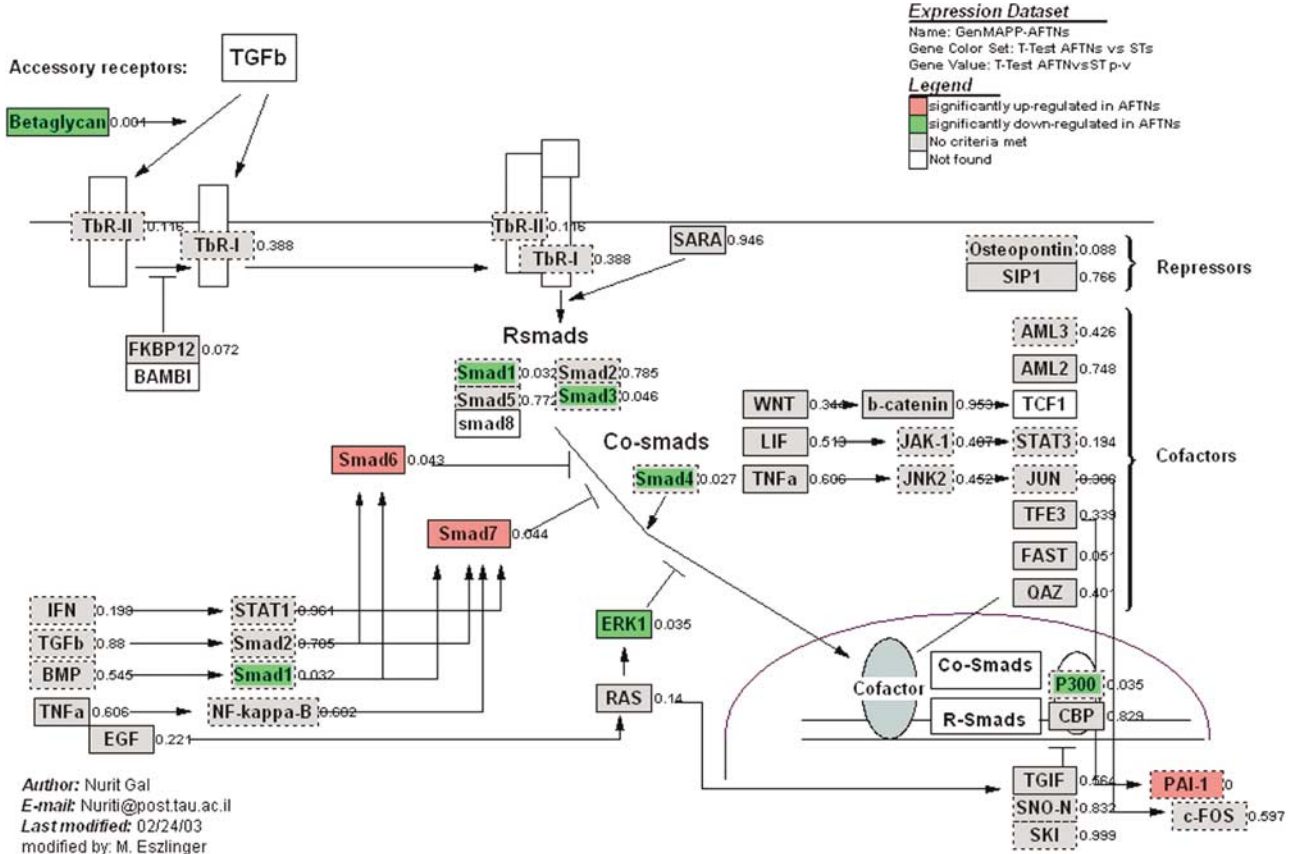


Figure 3 Diagram of the TGF- β signaling cascade from GenMAPP. Red colored boxes indicate significantly increased expression, whereas green colored boxes indicate a significantly decreased expression in AFTNs in comparison to STs. Beside the gene boxes, the P-value is given

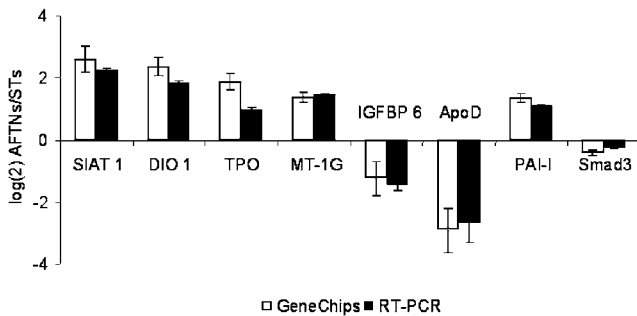


Figure 4 Comparison of the differential gene expression of eight genes determined by GeneChip analysis and real-time RT-PCR. The graph shows a high correlation between the GeneChip data (white bars) and the results of the real-time RT-PCR (black bars)

Thyroid-specific genes

Not unexpectedly, we found two upregulated genes encoding proteins that are involved in thyroid hormone metabolism: thyroid peroxidase and type I iodothyronine deiodinase. In contrast to DIO 1, we did not find altered expression of DIO 2 and 3 in AFTNs in comparison to their STs. DIO 1 converts T₄ into the active thyroid hormone T₃ and releases one iodide residue into the thyroid epithelial cell. This iodide

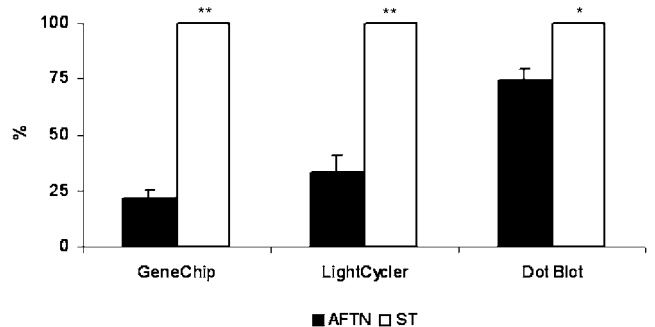


Figure 5 TGF- β receptor type III mRNA- and protein expression in four AFTNs (GeneChip, Dot Blot) and in 10 AFTNs (LightCycler) in comparison to their ST, respectively. GeneChip and LightCycler analysis show a significant downregulation of TGF- β III receptor mRNA in AFTNs in comparison to their STs (**P<0.01). Dot blot analysis revealed a significantly lower expression of TGF- β III receptor protein in AFTNs than in their STs (*P<0.05)

should be instantly available for TG iodination. An increase of TPO and DIO 1 would therefore increase the efficacy of iodine consumption, which could be compensatory in iodine deficiency.

SIAT 1 shows a very consistent increase of expression in AFTNs versus STs. Like for DIO 1, we also observed

an increased SIAT 1 mRNA expression after TSH stimulation of primary thyroid cells (data not shown). Sialylation is known to be one kind of protein modification influencing the biological properties of its targets. Most important, Oda *et al.* (1999) investigated the carbohydrate residues on the recombinant human TSHR with lectins of different specificity. They found that the formation of the TSH-binding site on the TSHR is dependent on complex post-translational modifications of the peptide chain in which carbohydrate residues play an important role. In particular, acquisition of complex-type sugar residues by the TSHR appears to be an important requirement for the formation of the TSH-binding site and the surface expression of the receptor. Our findings of increased SIAT 1 mRNA expression in AFTNs, together with an increased cell surface expression of the TSHR after SIAT 1 cotransfection, which in turn increases TSH radioligand binding, underlines the importance of post-translational modifications. Together with a TSH-dependent stimulation of SIAT 1 expression (data not shown), SIAT 1 action on TSHR forms a self-stimulatory loop that very likely amplifies TSHR signaling in AFTNs. Further experiments have to clarify the relevance of increased SIAT 1 expression in normal and diseased thyroid tissue. Moreover, they have to show the consequences of TSHR sialylation by SIAT 1 on the downstream cAMP- and IP-formation.

GenMAPP analysis

The analysis of our GeneChip data by GenMAPP revealed a distinctly changed pattern of gene expression of the TGF- β signaling pathway between AFTNs and their corresponding STs. Already in two former studies, we could identify differences in the expression of genes associated with the TGF- β signaling between AFTNs and their normal ST on protein- and mRNA-level. The investigation of TGF- β 1 by ELISA revealed a significantly decreased TGF- β 1 level in AFTNs in comparison to their STs (Eszlinger *et al.*, 2001a) and a recent investigation of 588 genes by cDNA expression array showed a significantly decreased expression of the type III TGF- β receptor (betaglycan) in AFTNs (Eszlinger *et al.*, 2001b). In addition, the differential expression of the type III TGF- β receptor was previously confirmed by real-time RT-PCR of 10 AFTNs and their ST (Eszlinger *et al.*, 2001b). Moreover, we also found a significantly decreased TGF- β receptor type III protein expression in AFTNs in comparison to their STs. The results of the present study are in line with the finding of a decreased expression of the TGF- β III receptor, which plays a more indirect role, since it delivers the ligand to the signaling receptors (Miyazono, 1997), increases TGF- β binding to the signaling receptors and enhances the cell responsiveness to the ligand (Lopez-Casillas *et al.*, 1993; Miyazono, 1997). Furthermore, the present study reveals a decreased expression of the downstream signaling molecules R-Smad1 and 3, which are directly phosphorylated by the type I TGF- β receptor (for a review, see Lutz and Knaus, 2002). Phosphorylated

R-Smads accumulate in the nucleus as heteromeric complexes with the Co-Smad, Smad4, for which expression in AFTNs is also decreased. In contrast, inhibitory Smads, like Smad6 and 7, show an increased expression in AFTNs. They counteract the effects of the R-Smads and thus antagonize TGF- β signaling. Moreover, p300, a transcriptional coactivator that acetylates core-histones and loosens the nucleosomal structure and allows transcription factors to access the general transcription machinery is characterized by a decreased expression in AFTNs, too. In spite of the fact that our study is focused on the mRNA expression levels of the genes, whereas the signal transduction of the mentioned cascades occurs mainly by post-translational modifications (e.g. phosphorylation), the pattern of gene expression of components of the TGF- β signaling cascade indicates a suppression of signaling via this cascade in AFTNs. This could also be one mechanism that restricts malignant transformation in these benign tumors (Grande *et al.*, 2002).

An interaction between the TGF- β signaling cascade and the EGF cascade has been shown (Kretzschmar *et al.*, 1997). Phosphorylation of four Erk consensus sites located in the linker region of Smad1 results in prevention of nuclear accumulation of Smad1. Moreover, oncogenic Ras was determined to initiate the same response but to produce stronger effects compared to EGF (Kretzschmar *et al.*, 1999). Nevertheless, our data do not show differentially expressed genes in the EGF signaling cascade with exception of Erk1, which would suggest an interaction of these cascades in AFTNs.

Inactivation of the TGF- β 1 cascade could be due to the constitutively activated TSHR signaling cascade. This assumption is supported by findings of Gärtner *et al.* (1997), who could show a decreased expression of TGF- β 1 mRNA after TSH stimulation of thyrocytes. Moreover, TGF- β 1 has been shown to inhibit iodine uptake, iodine organification and thyroglobulin expression (Pang *et al.*, 1992; Taton *et al.*, 1993), as well as cell proliferation in different thyroid cell culture systems (Tsushima *et al.*, 1988; Colletta *et al.*, 1989; Grubeck-Loebenstein *et al.*, 1989; Depoortere *et al.*, 2000). In the light of these findings, our results suggest that inactivation of TGF- β signaling is a major requisite for increased proliferation in AFTNs (Krohn *et al.*, 1999).

Cluster analysis

The hierarchical cluster analysis indicates that genes belonging to the metallothionein family, such as metallothionein 1A, 1E, 1F, 1G, 1H, 3 and RNA helicase-related protein, cluster closely to one another each showing increased expression in AFTNs. Increased expression of these genes was also identified by empirical filtering and Westfall-Young procedure. Metallothioneins have been postulated to regulate synthesis, assembly, or activity of zinc metalloproteins, and to protect against reactive oxygen species (Coyle *et al.*, 2002). In AFTNs, with an increased thyroid hormone synthesis, the metallothioneins might contribute to the regulation of metalloproteins such as superoxide

dismutase. In this context, a protective function of metallothioneins against reactive oxygen species in AFTNs is conceivable. In contrast to our findings of an increased expression of several metallothioneins in AFTNs in comparison to normal STs, Huang *et al.* (2001) described a decreased expression of the metallothionein mRNA-cluster in papillary thyroid carcinomas. This could be due to the decreased thyroid hormone synthesis in papillary carcinomas in comparison to normal thyroid tissue.

The Rap1 GTPase activating protein, for which mRNA is increased in AFTNs, specifically stimulates GTP hydrolytic activity of the G protein Rap1 (Zhang *et al.*, 1990). Interestingly, a recent study of Ribeiro-Neto *et al.* (2002) indicates that Rap1 is able to transduce the mitogenic cAMP signal while maintaining differentiation in thyroid epithelial cells. Vanvooren *et al.* (2001) investigated the role of Epac and Rap1 in the development of cold thyroid nodules by sequencing, but they found no mutations in these genes indicating that the cAMP-Epac-Rap1 pathway is not a major target for mutations in the development of cold thyroid nodules. However, our expression data suggest the participation of Rap1 in the signal transduction in AFTNs and further investigations, preferentially at the protein level are necessary to understand the role of Rap1 in nodular development.

Differential gene expression in AFTNs with and without TSHR mutations

Since we found a high concordance between the three applied methods of GeneChip analysis, we restricted the comparison of AFTNs with and without a TSHR mutation to the hierarchical clustering. In general, the comparison of the differential gene expression pattern in AFTNs with and without a mutation in the TSHR indicates a number of genes, which are part of different signaling pathways, such as GRK 2, RGS 4 and 6, Rap2, PAK 1 and 2, JAK 1 and protein kinase C. These findings indicate strong differences in the signal transduction of AFTNs caused by constitutively activating mutations in the TSHR compared to AFTNs without TSHR mutations, but further studies have to focus on differential protein expression of these genes.

In conclusion, our data show distinct differences of gene expression in AFTNs with and without a TSHR mutation and in their corresponding normal ST. Particularly, the TGF- β signaling cascade is characterized by strong changes in its pattern of gene expression between AFTNs and their ST, which might be due to the constitutively activated cAMP cascade. Moreover, the influence of sialylation of the TSHR onto its ligand binding and cell surface expression might be the scaffold of a new regulatory loop for TSH receptor activity. This could be an important link in the regulation of thyroid hormone metabolism in AFTNs and a promising start to understand the complex alterations of signal transduction in AFTNs through analysis of large patterns of gene expression.

Materials and methods

All AFTNs were identified by ultrasound and scintigraphy. All preoperatively identified nodules were also identified at surgery and postoperatively characterized by histology according to the WHO criteria (Hedinger, 1988). Somatic TSH receptor mutations in the hot nodules were previously determined by denaturing gradient gel electrophoresis and subsequent direct sequencing of the positive PCR fragments (Trulzsch *et al.*, 2001). AFTNs without a TSHR mutation were screened for mutations in the exons 7–10 of the $G_{s\alpha}$ protein by direct sequencing (forward primer: 5'-agt tgg caa att gat gtg agc-3', reverse primer: 5'-tct cta taa aca gtg cag acc-3'). However, no mutations in the $G_{s\alpha}$ protein were found. Informed consent for the analysis was given by the patients.

RNA isolation

Total RNA was isolated from 15 AFTNs, their corresponding surrounding normal tissue (ST) using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. Later, the total RNA was purified with RNeasy kits (Qiagen, Hilden, Germany) according to the RNA clean-up protocol.

The quality and quantity of the total RNAs was examined on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) using the RNA 6.000 LabChip Kit (Agilent Technologies) according to the manufacturer's instructions.

Microarray analysis

A weight of 10 μ g of total RNA was used to prepare double-stranded cDNA (Superscript II, Life Technologies, Gaithersburg, MD USA) primed with oligo-dT containing a T7 RNA polymerase promoter site (Genset SA, Paris, France). cDNA was purified by phenol-chloroform extraction before *in vitro* transcription using the ENZO BioArray RNA transcript labeling kit (Affymetrix, Santa Clara, CA, USA) to synthesize cRNA. After the *in vitro* transcription, unincorporated nucleotides were removed using the RNeasy kit (QIAGEN, Hilden, Germany). The cRNA was fragmented and hybridized to Affymetrix GeneChip U95Av2.

The washing, staining, and scanning of the probe array was performed according to the manufacturer's instructions.

Data analysis

Affymetrix GeneChip data representing approximately 10 000 full-length genes were extracted from fluorescence intensities and were scaled in order to normalize data for interarray comparison.

To detect differentially regulated genes, different methods of data analysis were used:

- (a) *Empirical filtering*: We manually selected genes, which were characterized by the Affymetrix software by 'increased' or 'decreased' and a signal log ratio greater than 0.585 or lower than -0.585 (i.e. these genes are at least 1.5-fold higher or lower expressed in the AFTN than in the corresponding ST) in 13 out of 15 patients, respectively.
- (b) *Statistical analysis*: In order to avoid a high rate of false positive results in this multitude of tests for the different genes, we added so-called multiple test procedures. After a logarithmic transformation of the data, we computed adjusted *P*-values for each gene according to the Westfall-Young procedure, which imbeds a *t*-test into a permutation procedure (Westfall and Young, 1993).

- (c) *Cluster analysis*: Hierarchical cluster analysis using the correlation metric and average linkage was performed by means of the Cluster and TreeView software (<http://www.rana.lbl.gov>) (Eisen *et al.*, 1998). Before clustering, two gene sets were created. Set 1 (725 genes) was trimmed of genes that showed a differential expression for AFTNs and STs in less than five out of 15 patients. Set 2 (714 genes) contains genes that show a significant ($P < 0.05$) differential expression between AFTNs with and without TSHR mutations. Although loss of some information is possible, trimming the number of genes decreases the influence by genes with little or no differential expression.

GenMAPP

GenMAPP (Gene Microarray Pathway Profiler, downloaded from www.GenMAPP.org) complements and extends available statistical and clustering algorithms (Dahlquist *et al.*, 2002). In contrast to statistical filters and pattern-finding algorithms (e.g. hierarchical clustering) GenMAPP analyses the gene expression changes in the context of known biological pathways. Gene expression data including P -values were imported into GenMAPP in a comma-separated-value (CSV-file) format. GenMAPP converts the expression data into a data set that can then be viewed on any MAPP with any number of color-coding criteria sets. The following color-coding criteria were defined in AFTNs: significantly ($P < 0.05$) upregulated genes are colored red and significantly down-regulated genes are colored green.

Real-time RT-PCR

The quantification of several differentially expressed genes by real-time RT-PCR was performed using a LightCycler (Roche, Mannheim, Germany) as previously described (Eszlinger *et al.*, 2001b). The nucleotide sequences of the primers and PCR conditions are available on request. The differential expression of the investigated genes was calculated as the log(2)-ratio AFTN/ST. The determined ratios were also normalized to the ratio of the housekeeping gene β -actin.

Dot blot

Protein extraction for dot blot was performed as previously described by Vicencio *et al.* (2002). The protein concentration of the whole tissue lysates was measured using the BCA Protein Assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Weights of 100 μ g of the whole tissue lysate of four AFTNs and their ST, 0.5 μ g of the recombinant human TGF- β receptor type III (R&D Systems, Minneapolis, MN, USA) (positive control) and 2 μ g of BSA (negative control) were blotted onto a nitrocellulose membrane. The membrane was blocked with 5% dry milk for 1 h at room temperature. Afterwards, it was incubated with goat anti-human TGF- β receptor type III antibody (1 : 500; R&D Systems, Minneapolis, MN, USA) at 4°C overnight in Tris-

buffered saline + 0.1% Tween20 (TBST) + 5% BSA, followed by three washes in TBST. Finally, the membrane was incubated with a peroxidase conjugated rabbit-anti-goat IgG (Chemicon International Inc.) for 1 h at room temperature and then the immunoreactivity was visualized by enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce).

Cell culture and transfection

The influence of SIAT 1 expression on functional properties of the TSHR was investigated in cell culture. COS-7 cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in a humidified 5% CO₂ incubator. Cells were transiently transfected in 12-well plates (1 × 10⁵ cells per well) with 1 μ g DNA/well (TSHR-pSVL (Libert *et al.*, 1989) and SIAT 1-pcDNA3.1/V5-His) using the FuGENE reagent (Roche, Basel, Switzerland).

Radioligand binding assay

Competitive binding studies were performed as previously described (Wonerow *et al.*, 1998). To determine the TSH radioligand binding at a similar level of TSHR cell surface expression, we balanced the effect of SIAT 1 cotransfection on TSH receptor expression compared to empty pcDNA by cotransfecting the human V2 vasopressin receptor together with the TSH receptor and the SIAT 1 (Schoneberg *et al.*, 1996; Neumann *et al.*, 2001). In these experiments, SIAT 1 cotransfection did no longer affect TSH radioligand binding, which indicates that increased cell surface expression in response to SIAT 1 overexpression is the mode of action.

FACS analysis

FACS analysis was performed as previously described (Neumann *et al.*, 2001). Receptor cell surface expression was determined by the fluorescence intensity, whereas the percentage of signal positive cells indicates the transfection efficiency.

ELISA

The cell surface expression of the TSHR determined by FACS analysis was confirmed by ELISA as previously described (Wonerow *et al.*, 2000).

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