

Aus der Klinik für
Allgemein-, Viszeral- und Transplantationschirurgie
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**Identifikation differentiell exprimierter Gene
in papillären Schilddrüsenkarzinomen
mit und ohne Rearrangements
der Tyrosinkinase-Rezeptoren RET und/oder NTRK1**

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1. Zusammenfassung

1.1. Einleitung

Schilddrüsenmalignome treten in Deutschland mit einer Inzidenz von drei Neuerkrankungen pro 100.000 Einwohner und Jahr auf (Varianz 0,8 bis 5 für Männer, 1,9 bis 19,4 für Frauen) [1]. Frauen sind etwas häufiger als Männer betroffen. Die Karzinome werden zunächst nach histomorphologischem Bild in differenzierte und undifferenzierte Karzinome unterteilt. Zu den differenzierten werden das am häufigsten auftretende papilläre (papillary thyroid carcinoma - PTC, eher 65-80%) und das follikuläre Schilddrüsenkarzinom (follicular thyroid carcinoma - FTC, 5 - 15%) gerechnet [2], die beide von den hormonproduzierenden Follikelepithelzellen ausgehen. Die undifferenzierten oder anaplastischen Karzinome (undifferentiated thyroid carcinoma - UTC) treten deutlich seltener auf (~10%). Neuerdings werden die wenig differenzierten Schilddrüsenkarzinome noch als Zwischengruppe zwischen den differenzierten und anaplastischen Schilddrüsenkarzinomen unterschieden. Ein typischer Vertreter ist das insuläre Schilddrüsenkarzinom. Dagegen leitet sich das medulläre Schilddrüsenkarzinom (medullary thyroid carcinoma - MTC) von den parafollikulären C-Zellen ab. Es ist ähnlich selten wie das UTC (~10%), allerdings gibt es familiäre Häufungen (z.B. im Rahmen der Multiplen Endokrinen Neoplasien, MEN 2A und MEN 2B).

Papilläre Schilddrüsenkarzinome sind im Allgemeinen mit einer guten Prognose assoziiert. Dennoch beinhaltet die Gruppe der papillären Schilddrüsenkarzinome ein breites Spektrum unterschiedlich schnell progredienter und metastasierender Tumoren. Seit mehr als einem Jahrzehnt wird daher zunehmend nach den molekulargenetischen Grundlagen ihrer unterschiedlichen biologischen Verhaltensweisen geforscht. Es sind z.B. Veränderungen des Rezeptors für Thyroidea stimulierendes Hormon (TSH) beschrieben worden, doch bedarf es zur malignen Transformation scheinbar mehrerer zusammentreffender Alterationen. In schlecht differenzierten und anaplastischen Schilddrüsenkarzinomen sind beispielsweise die Tumorsuppressorgene TP53 und das Retinoblastomgen (Rb) häufig inaktiviert.

Mutationen des RET-Protoonkogens sind kausal für das hereditäre MTC. In PTCs konnten dagegen spezifische Neukombinationen der Tyrosinkinase-Region des RET-Rezeptors mit Promotor-Regionen ubiquitärer Gene nachgewiesen werden. Eine solche RET-Chimäre wurde bei einer Transfektion erstmalig als Kombination aus dem RET-Gen und einem Promotorgen identifiziert, woher auch die Bezeichnung RET für *RE*arranged

during Transfection rührt. Das breite wissenschaftliche Interesse wurde den Neukombinationen aber erst zuteil, nachdem solche Veränderungen in 20 – 30% papillärer Schilddrüsenkarzinome nachgewiesen wurden und darüber hinaus die Spezifität für den papillären Histotyp beobachtet wurde [3, 4]. In jüngster Zeit wurden RET-Rearrangements auch in nicht-follikulär-differenzierten oxyphilen Schilddrüsenkarzinomen beschrieben [5]. Das RET-Gen büßt durch eine Chromosomenveränderung zwar Teile seiner Sequenz ein, die erhaltene intrazelluläre Tyrosinkinase-Region erhält jedoch einen aktivierenden Promotor, der die Expression der RET-Chimäre bewirkt. So führt es zu einer unphysiologischen Erhöhung der Tyrosinkinase (TK)-Aktivität in den Follikelzellen, die dieses Genprodukt im Erwachsenenalter normalerweise nicht mehr exprimieren. Bis heute wurde von insgesamt zwölf verschiedenen Partnergenen berichtet, mit denen mindestens 16 unterschiedliche RET-Hybridonkogene gebildet werden. Sie werden mit RET/PTC 1, 1L, 2, 3, $\Delta 3$, 4-13 bezeichnet. Einige RET/PTC Varianten entstehen durch variable Intron-Bruchpunkte in RET (RET/PTC 4) beziehungsweise den Partnergenen (RET/PTC 1long, $\Delta 3=3r2$ und 3r3) [6].

In den betroffenen Zellen wird durch die RET-TK ein Signalweg aktiviert, der über eine Ras-abhängige BRAF-Aktivierung die Zellproliferation und Invasion der Umgebung anregt. Eine Mutation entweder von RET, RAS, oder BRAF findet sich in 70% der PTCs [7].

NTRK1 (syn. TrkA) ist ein membranständiger TK-Rezeptor für den Nervenwachstumsfaktor. In Analogie zu RET entsteht das entsprechende Protoonkogen ebenfalls durch chromosomale Neukombinationen durch Vorschaltung eines Promotors vor die TK-Region des Rezeptors, deren einzelne Ausprägungen mit trk, TRK-T1, -T2 und -T3 bezeichnet werden. Auch hier trägt eine onkogen erhöhte TK-Aktivität zum malignen Verhalten der Tumorzellen bei. NTRK1-Neukombinationen scheinen durch ionisierende Strahlung begünstigt zu werden.

Man nimmt an, dass die Expression von RET bzw. NTRK1 ein frühes Stadium der Tumorigenese darstellt, aber nicht zwingend zur Entartung führt. Neuere Untersuchungen zeigen eine Korrelation des exprimierten Hybridonkogens mit dem biologischen Verhalten des Tumors: RET/PTC1 tragende Neoplasien verhalten sich z.B. in der Regel deutlich weniger aggressiv als solche, in denen sich RET/PTC3 nachweisen lässt [8, 9].

Die komplexe Erfassung der Bedeutung von RET und NTRK1 Chimären für die Entwicklung papillärer Schilddrüsenkarzinome (und auch oxyphiler Schilddrüsenkarzinome) wie auch die weitere Ergründung der Faktoren, die sich in nicht-chimären Tumoren auswir-

ken, steht demnach noch aus. Somit untersuchten wir eine Auswahl von PTC-Geweben mit und ohne entsprechende Neukombinationen mittels mRNA-Differential Display. Wir strebten hiermit die Identifikation von Genprodukten an, die zusätzlich zu den chimären Tyrosinkinaserzeptoren an einer malignen Transformation der Zellen beteiligt sein könnten bzw. die Progression dieser Tumoren beeinflussen.

1.2. Material und Methoden

Schockgefrorenes Gewebe von Primärtumor bzw. Lymphknotenmetastasen von 13 PTCs mit und ohne RET- und NTRK1-Neukombinationen und korrespondierendes normales Schilddrüsengewebe der Patienten wurden untersucht. Es wurden Gewebe von Tumoren ausgewählt, die sich in verschiedenen Punkten deutlich unterschieden: z.B. Vorhandensein einer Neukombination, histologisches Bild und biologisches Verhalten, Lebensalter der Tumordiagnose, TNM-Stadium und postoperativer Langzeitverlauf. Zur Qualitätskontrolle wurden die Gewebeblöcke, die zur Extraktion verwendet wurden, geteilt, und eine Hälfte histologisch untersucht. Gewebeblöcke mit geringem Tumorzellanteil wurden von der Untersuchung ausgeschlossen.

Nach Extraktion der totalen RNA, DNA-Elimination sowie reverser Transkription wurde die resultierende cDNA mittels PCR amplifiziert. Hierbei verwendeten wir eine Kombination aus einem sogenannten „anchored Primer“ und einem „arbitrary Primer“: Der anchored Primer wird aus einer Gruppe von drei „Poly-A“-Primern gewählt, bestehend aus 15 Adenin-Nukleotiden mit einem zusätzlichen Guanin- oder Cytosin- oder Thymin-Nukleotid, der sich daher immer am Poly-T-Ende der cDNA anheften wird, ohne jedoch einen großen Teil der Poly-T-Kette mit zu amplifizieren. Der mit sieben bis acht Basenpaaren (bp) sehr kurze arbitrary Primer weist eine ubiquitäre Sequenz auf. Dies führt zu einer ausreichenden Wahrscheinlichkeit, dass er sich auf sehr vielen 200 bis 600 bp langen cDNA-Fragmenten anheftet. Von den 12.000 (in Tumorzellen eher 14.000) unterschiedlichen mRNAs einer Zelle werden pro Primerkombination nur etwa 200 der PCR-Reaktion zugänglich. Multiple PCRs mit der Kombination von allen drei anchored mit den arbitrary Primern jedoch führen zu einer Amplifikation von einem großen Anteil der in der Probe vorhandenen cDNA in Fragmenten von wenigen hundert Basenpaaren. Zur Qualitäts- und Reproduzierbarkeits-Kontrolle wurde jede Kombination doppelt angesetzt.

In der folgenden Polyacrylamid-Gel-Elektrophorese (PAGE) zeigten sich Expressionsunterschiede für die mRNAs im Bandenmuster der verschiedenen Gewebe. Die gewonnenen cDNA-Amplifikate wurden zunächst auf einem automatischen Sequencer aufgetrennt, weil dieser eine hohe Detektionsgenauigkeit und digitale Bildbetrachtungsmöglichkeiten bietet. Das Gelbild entsteht hierbei nur virtuell, die cDNA-Fragmente passieren das Gel bei der mehrstündigen Elektrophorese vollständig und sind daher für weitere Analysen verloren. Bei der Auswertung des Gelbildes wurden die differentiell exprimierten Banden bestimmt und nur die Proben, welche die einzelnen differentiellen Fragmenten beinhalteten, wurden zum Zwecke der Bandenisolierung weiter bearbeitet. Eine erneute PCR-Amplifikation der betreffenden Probe mit speziell fluoreszenzmarkierten Primern wurde unter exakt gleichen PCR-Bedingungen durchgeführt. Eine zweite PAGE erfolgte mit einem sehr dünnen langen Gel, das mit hoher Spannung (3000V) betrieben wurde. Durch eine vorherige Behandlung der Glasplatten blieb das Gel beim Trennen der Platten nach der Elektrophorese intakt und die Banden konnten mittels Hochleistungs-Großformat-Fluoreszenzscanner detektiert und schlussendlich gezielt aus dem Gel ausgeschnitten werden. Nach Konditionierung und Qualitätsprüfung der gewonnenen Nukleotid-Sequenzen wurden diese kloniert und sequenziert. Mithilfe von NCBI-BLAST (Basic Local Alignment Search Tool, Version 2.0) wurden die Sequenzen identifiziert [10].

Die einzelnen Arbeitsschritte des mRNA Differential Display werden anhand der fotografischen Momentaufnahmen verdeutlicht (Bild 1, publiziert in [11]).

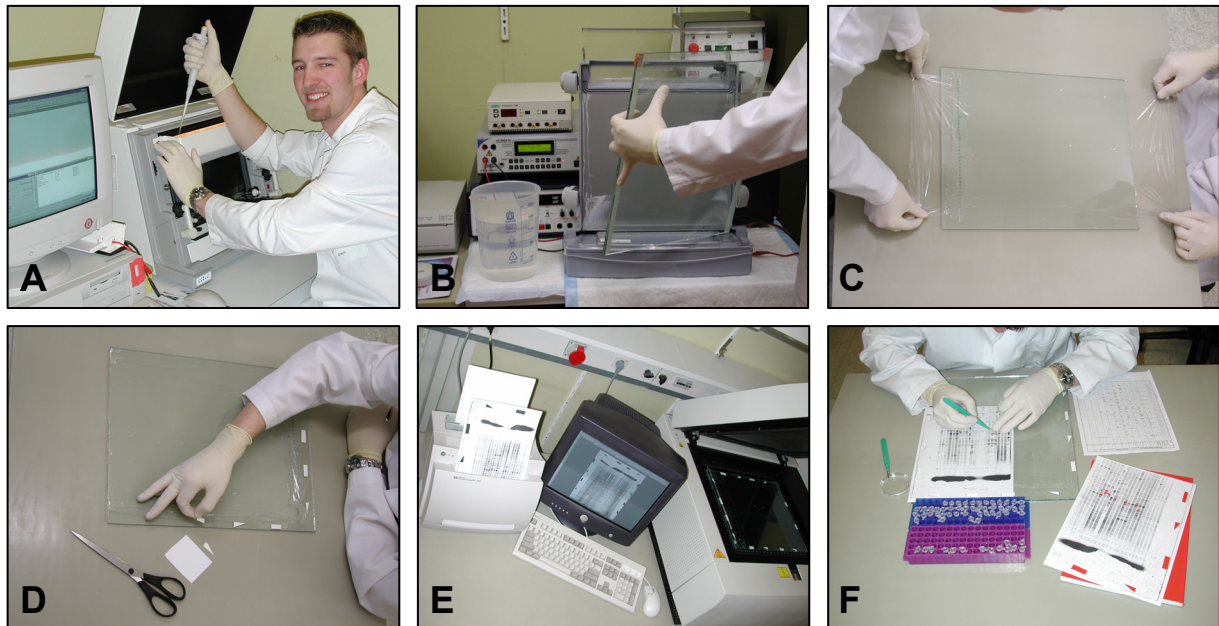


Bild 1: Vorgehen zur Gewinnung fluoreszenzmarkierter Transkripte: Proben differentiell exprimierter cDNA-Fragmente können nach der Elektrophorese im Sequencer (A) nicht mehr gewonnen werden. Daher wurde ein Aliquot der entsprechenden cDNA nach erneuter PCR mit fluoreszenzmarkiertem Primer (und sonst gleichen Bedingungen) erneut elektrophoretisch aufgetrennt, diesmal auf einem konventionellen Sequenzierungsgel (B). Das resultierende Gel wurde auf der Glasplatte belassen und mit dünner transparenter Folie abgedeckt (C), am Rand der Platte wurden fluoreszierende Markierungen angebracht (D). Das gesamte Gel wurde mit einem Fluoreszenzscanner erfasst und ein exakter 1:1 Papierausdruck erstellt (E). Bei korrekter Auflage auf das Gel ließ sich so die Position der Bande genau bestimmen, die das gesuchte cDNA-Fragment enthält (F). Die Bande wurde sorgfältig aus dem Gel ausgeschnitten und in ein sauberes Reaktionsgefäß überführt (F).

1.3. Ergebnisse

Insgesamt zeigten die durchgeführten cDNA-Elektrophoresen über 500 Bandenkombinationen, die auf unterschiedlich exprimierte mRNA-Transkripte in den parallel untersuchten Geweben hindeuteten. Von 276 isolierten Banden wurden 44 für die Reamplifikation ausgewählt, die bei 24 Transkripten erfolgreich verlief. Die Klonierung blieb für fünf Proben erfolglos, sodass letztendlich 19 Fragmente zur Auswertung kamen. Hierbei fanden sich für einige Tumore besonders viele differentiell exprimierte mRNA-Fragmente, während andere wenige Unterschiede zu den Vergleichsgeweben zeigten.

Drei Transkripte wurden mehrfach nachgewiesen: Thyroperoxidase (TPO), das 14-3-3 β /YWHAB Protein und ein aberrantes Homologon zur konstanten Region des Kappa-Leichtketten Fab-Fragments eines Immunglobulins. Aus besonders ausgeprägten Ban-

den ließen sich in einigen Fällen erwartungsgemäß Fragmente mit unterschiedlichen Basensequenzen klonieren. Ebenso können Banden parallel untersuchter verschiedener Gewebe, die exakt auf gleicher Höhe im Gel zu liegen scheinen, Transkripte unterschiedlicher Sequenz enthalten. Im Falle eines Fragments, das sich in schlecht differenziertem Tumorgewebe im Vergleich zu gut differenziertem Tumorgewebe überexprimiert zeigte, fand sich eine Basensequenz, die für Teile von zwei unterschiedlichen Proteinen kodierte. Es stellte sich heraus, dass die Genloci beider Proteine auf Chromosom 2p25.1 liegen, und zwar komplementär auf den gegenüberliegenden DNA-Strängen mit einer gewissen Überlappung am 3'-Ende jedes dieser Gene.

Außerdem wurden weitere bekannte oder noch unbekannte Genprodukte identifiziert, die differierende Expression in normalem gegenüber tumorösem Schilddrüsengewebe, Lymphknotenmetastasen gegenüber dem Primärtumor oder unterschiedlich differenzierten Tumoren zeigten. Dabei ließen sich auch Expressionsunterschiede nachweisen, die mit unterschiedlichen RET- oder NTRK1- Neukombinationen assoziiert waren.

1.4. Diskussion

Die Diagnostik solider Tumore auf molekularer Ebene stützt sich auf den Nachweis genetischer Marker oder die veränderte Expression von mRNAs, die für maligne Gewebe oder einen bestimmten Tumortyp kennzeichnend ist. Ein weiterer wichtiger Aspekt ist, dass solche Charakteristika, sind sie erst einmal sicher festgestellt worden, Türen zu neuen Therapieansätzen öffnen können. Hierfür besteht in der Behandlung von Patienten mit schlecht differenzierten PTCs sowie PTCs, die sich aus anderen Gründen für die bisher etablierten Maßnahmen therapierefraktär zeigen, dringender Bedarf.

In der Literatur sind bereits viele Versuche mit mannigfaltigen Techniken beschrieben, solche Tumorcharakteristika zu identifizieren. Die vorliegende Arbeit gehört dabei zu den sehr wenigen Expressionsanalysen in PTCs, die via totalem mRNA Differential Display als offenes Screening konzipiert ist. Die meisten anderen Arbeitsgruppen benutzten gezielte Ansätze, die z.B. mRNAs mit hohem Umsatz oder mitochondriale Bestandteile nachweisen können, oder auch Microarrays, die auf krebsspezifische Expressionsmuster ausgerichtet sind. Nur eine Studie betrachtete die genetischen Grundlagen der PTCs, beispielsweise die Onkogene BRAF, RET und NTRK 1, in größerer Breite und wies auf assoziierte Genexpressionsunterschiede hin [12].

Ein Vorteil unserer Methode liegt somit auf der Hand: es sind potenziell alle mRNAs der Tumorgewebe - je nach Primerkombination - untersuchbar, wodurch insbesondere noch unbekannte herauf- oder herunterregulierte Transkripte miterfasst werden. Dies bestätigte sich in unseren Ergebnissen. Zusätzlich lässt sich unsere Methode mit deutlich geringerem finanziellem und personellem Aufwand als vor allem die Microarray-Untersuchungen durchführen.

Die Genauigkeit und Reproduzierbarkeit der Methode geht aus mehreren Tatsachen hervor: Bei der hohen Zahl an unterschiedlichen Primerkombinationen zeigten sich für die jeweils parallel bearbeiteten identischen Ansätze jeder mRNA-Probe in fast 100% der Fälle identische Bandenmuster. Weiterhin zeigten sich bestimmte Expressionsmuster selbst in Reaktionen mit anderen Primern erneut. Außerdem konnte die Thyroperoxidase (TPO) mehrfach in normalem Schilddrüsengewebe und hoch differenziertem Tumorgewebe nachgewiesen werden, wohingegen sie in mäßig oder schlecht differenziertem Tumorgewebe häufig fehlte. Dies fungiert de facto als „Positivkontrolle“ und hebt die Plausibilität der Ergebnisse hervor. In einer weiteren, parallelen Untersuchung an medullären Schilddrüsenkarzinomen unserer Arbeitsgruppe stellte sich die Methode ebenfalls als funktionierend heraus [11].

Unter den im Karzinomgewebe hochregulierten Genprodukten fand sich z.B. das 14-3-3 β /YWHAB-Protein. Nach bisherigen Erkenntnissen spielt es eine Rolle in der Regulation des Zellzyklus, der Apoptose und Mitose. Es zeigte sich in Tumorzelllinien von Maus und Ratte überexprimiert, seine tumorigene Wirkung wurde an Zellen eines hepatozellulären Karzinoms der Ratte nachgewiesen. Weiterhin interagiert 14-3-3 β mit dem Rezeptor für den insulinähnlichen Wachstumsfaktor I (IGFIR), dessen hohe Expression besonders in Schilddrüsentumoren mit aggressivem Verhalten gezeigt wurde. Möglicherweise werden also IGFIR und die nachfolgende Kaskade durch 14-3-3 β reguliert. Es ist daher gut vorstellbar, dass es sich auch auf die Penetranz von Hybridgenen der Tyrosinkinase-rezeptoren RET und NTRK 1 auswirkt. 14-3-3 β scheint ein neues Onkogen zu sein, das in Kooperation mit anderen Onkogenen eine Zelle maligne transformieren kann, und unsere Ergebnisse legen nahe, dass es eine Rolle in der Entstehung von PTCs spielt. Da es regulierend in den Zellzyklus eingreift, stellt es einen möglichen Angriffspunkt für neue medikamentöse Therapien entdifferenzierter PTCs dar.

Rab27a fand sich in Tumoren mit und ohne TK-Neukombination unterexprimiert. Es hat eine Funktion im Vesikeltransport. Das verminderte Vorkommen könnte ein Hinweis auf eine Entdifferenzierung und einen Verlust der Hormonsekretion sein.

Außerdem wurde die V-ATPase C2 Untereinheit in mäßig bis schlecht differenzierten Tumoren überexprimiert nachgewiesen. Sie ist Teil einer membranständigen Protonenpumpe, die in Tumoren mit schlechter Vaskularisierung und daher Azidoseneigung möglicherweise einen physiologischen pH aufrechterhält.

Mit dem protein-disulfide isomerase-related protein (hPDIR) und dem Homolog zur konstanten Region einer Immunglobulin(Ig)-Kappa-Leichtkette wurden zwei weitere Transkripte isoliert, über deren Rolle in der Karzinomprogression z.Zt. nur vage Aussagen gemacht werden können. Es geht allerdings aus unseren und auch den Ergebnissen einer anderen Arbeitsgruppe hervor [13], dass das Ig-Homolog durchaus ein Produkt der Tumorzellen sein kann und nicht zwingend aus Zellen des Immunsystems stammen muss.

Letztendlich konnten mehrere Genprodukte mit potenzieller tumorigener Wirkung in plausiblen Expressionsmustern nachgewiesen werden, aber wegen der z. Zt. noch relativ kleinen Zahlen an untersuchten Geweben ist noch keine Aussage dazu zu treffen, inwieweit die RET- und NTRK1-Neukombinationen diese beeinflussen. Die Methode scheint jedoch geeignet zu sein, bei Fortführung der Untersuchungen Erkenntnisse zu liefern, die zu einem besseren Verständnis der Entwicklung von papillären Schilddrüsenkarzinomen führen werden. Daher planen wir, die vielen noch nicht untersuchten cDNA-Fragmente des im Rahmen dieser Arbeit erstellten Panels mittels einer quantitativen real-time PCR (LightCycler) weiter auszuwerten. Diese Technik stand uns für diese Arbeit noch nicht zur Verfügung und ermöglicht die Prozessierung von Proben in wesentlich kürzerer Zeit unter Einsparung von Arbeitsschritten zur Produktdarstellung mittels PAGE und zur Produktquantifizierung. So werden mit steigenden Ergebniszahlen valide statistische Aussagen zu den Zusammenhängen genetischer Gegebenheiten und Expressionsunterschieden mit dem Tumorverhalten in vivo und Krankheitsverläufen möglich werden und damit den Weg für neue diagnostische und therapeutische Verfahren bereiten.

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Identification of differentially expressed genes in papillary thyroid carcinomas with and without rearrangements of the tyrosine kinase receptors RET and/or NTRK1.

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Identification of Differentially Expressed Genes in Papillary Thyroid Carcinomas With and Without Rearrangements of the Tyrosine Kinase Receptors RET and/or NTRK1¹

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Background. The transforming capacities of *RET* and/or *NTRK1* chimeric oncogenes as well as the molecular background of non-rearranged papillary thyroid carcinomas (PTCs) remain to be elucidated. To assess altered gene expression, we examined PTCs with and without tyrosine kinase receptor rearrangements by mRNA differential display (DD).

Materials and methods. Six of 13 PTCs examined harbored *RET* chimeras (3× *RET*/PTC1, 1× *RET*/PTC3) and/or *NTRK1* chimeras (2× *trk*, 1× *TRK-T3*, 2 unknown *TRK* hybrids). The method of DD analysis was refined by a novel fragment-recovery technique using a high-performance fluorescence scanner.

Results. Of 500 up- or down-regulated mRNA transcripts, 19 selected fragments were recovered, cloned, sequenced, and identified. The accuracy and high degree of reproducibility of the method was demonstrated. Differential expression of gene products with potential association to cell proliferation or tumor progression was observed, such as 14-3-3beta and Rab27a. Moreover, several gene products with unknown functions were demonstrated in PTCs bearing *RET* or *NTRK1* hybrids versus rearrangement-negative PTCs, including a homologue of the Ig kappa light chain constant region.

Conclusions. Candidate transcripts with presumed tumorigenic potential in other solid tumors may

prove to be relevant in the progression of PTCs, too. Most promising is the isolation of several differentially expressed, yet unknown, genes that may open new insights in the pathogenesis or progression of PTC. © 2006 Elsevier Inc. All rights reserved.

Key Words: papillary thyroid carcinoma; differential gene expression; chromosomal rearrangements; genetic alterations; mRNA differential display.

INTRODUCTION

Papillary thyroid carcinomas (PTCs) are the most frequent thyroid malignancies. Although the tumors are generally associated with a favorable prognosis, a wide spectrum of biological behavior can be observed that is most likely based on varying genetic backgrounds of the respective neoplasms.

Different genetic alterations have already been described in thyroid tumors: alterations of the thyroid-stimulating hormone receptor (TSHR) and of the membrane-bound G protein—both leading to a constitutive activation of the adenylate cyclase-protein kinase A (PKA) pathway—have been identified in benign neoplasms, especially in hyperfunctioning adenomas, as well as in congenital hyperthyroidism and in a subset of differentiated thyroid carcinomas [1, 2]. However, additional molecular alterations seem to be required for malignant transformation of the thyrocyte following activation of the aforementioned oncogenes. The retinoblastoma (Rb) gene and especially the TP53 gene are tumor-suppressor genes that are inactivated in some thyroid cancers with predominantly poorly differentiated phenotype [3, 4] (Fig. 1).

Starting about 15 years ago, rearrangements of the *RET* proto-oncogene were detected in papillary carci-

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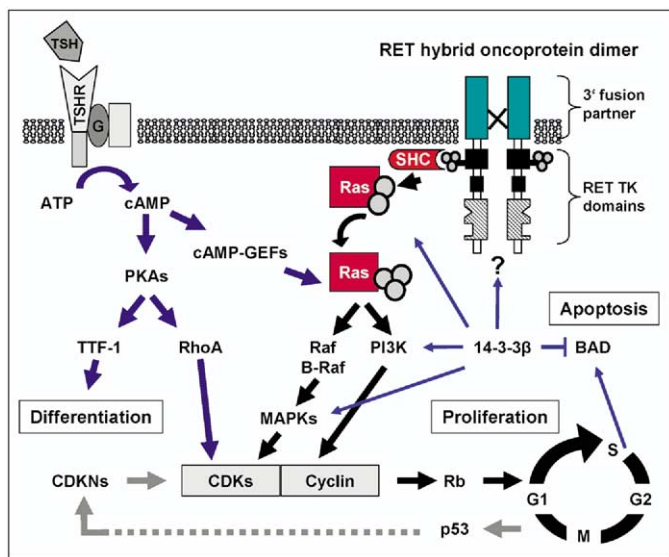


FIG. 1. Selected signaling and modulating pathways in the oncogenesis of papillary thyroid carcinomas (PTCs) are illustrated. The physiological proliferation of the thyroid cell is initiated through activation of the TSH receptor (TSHR). Following binding of the thyroid growth hormone TSH, G-protein coupled to the receptor is activated leading to a well-established signal cascade involving protein kinases A (PKAs). Thus, the hormone signal is transmitted to the nucleus where the transcription of proteins necessary for the cell-cycle progression is initiated. As a result, the cyclin-dependent kinases (CDKs) are activated that “flip the switch” in favor of the progression to DNA synthesis and mitosis. Intact feedback loops controlling cell proliferation and tissue integrity—here partially depicted by the action of p53 and the inhibitors of the cyclin-dependent kinases (CDKs)—will limit the number of cell divisions. Alterations of TSHR or G-protein, resulting in increased mitoses, are predominantly described in thyroid adenomas. Similarly, unphysiological activation of the hybrid RET receptor—following chromosomal rearrangement with the promoter of a fusion partner gene (RET/PTC1-12)—leads to tyrosine kinase (TK) activity, which initiates cell proliferation through the Ras pathway involving Raf, B-Raf, and phosphatidylinositol 3-kinase (PI3K). Mitogenic gain-of-function mutations of the *RAS* oncogene or of *BRAF* are also reported for PTCs. 14-3-3β protein (whose mRNA transcript we found to be overexpressed in carcinomas) interacts with proteins of several levels of the Ras signal cascade. Hypothetically, up-regulation of 14-3-3β will result in increased mitosis by activation of Ras or its downstream pathway and will halt apoptosis by inhibition of the pro-apoptotic protein BAD. (Color version of figure is available online.)

nomas of the thyroid gland and the specificity of these findings for PTCs were proven [5–7]. Only solitary observation of *RET*-rearranged follicular thyroid carcinomas (FTCs) are published [8]. The oncogenic activation of a chimeric *RET* gene is the result of translocations between the *RET* locus 10q11.2 and another chromosome or the result of an inversion of chromosome 10. Thus, the tyrosine kinase (TK) domain of the *RET* proto-oncogene is fused to the amino terminus of one of several ubiquitously expressed genes which donate the promoter region for the resulting chimera. The replacement of the extracellular domain of the *RET* receptor in somatic cells leads to constitutive and

ectopic expression of the resulting hybrid oncoproteins which causes an increased tyrosine kinase activity in tissues physiologically lacking expression of *RET*. To date, 11 different fusion partner genes are reported forming at least 15 different *RET* hybrid oncogenes, designated RET/PTC 1, 1L, 2, 3, Δ3, 4–12 [5, 9; Imkamp and Musholt, submitted for publication, 2006]. Some RET/PTC variants are formed by variable intronic break-points in *RET* (RET/PTC 4) or in the partner genes (RET/PTC 1long, Δ3 = 3r2 and 3r3), respectively.

In a linear signaling cascade, the rearranged *RET* receptor induces a Ras-dependent activation of B-raf, stimulating cell proliferation and matrix invasion of the respective thyrocytes [10] (Fig. 1). Congruously, either (1) a RET/PTC rearrangement or (2) an activating point mutation within *RAS* or (3) a *BRAF* oncogene mutation can be detected in up to 70% of papillary tumors of the thyroid gland [11, 12]. Combined genetic alterations of *RET*, *RAS*, and *BRAF* have only rarely been described [13].

Comparable to *RET*, the proto-oncogene encoding for the tyrosine kinase receptor *NTRK1* (synonymously designated *TrkA*), a transmembrane receptor for the nerve growth factor, is rearranged and oncogenically activated in PTCs, resulting in the chimeric oncogenes *trk*, *TRK-T1*, -2, -3 [14, 15].

Rearrangements of the TK domains of the receptors *RET* and *NTRK1*, respectively, have been observed in 0–61% of all non-radiation-induced PTCs and in up to 90% of patients with a history of radiation exposure (i.e., therapeutic external beam irradiation to the neck, Chernobyl-associated radio-iodine contamination, etc.) [14, 16, 17].

Expression of *RET* or *NTRK1* hybrid oncogenes is considered an early event in the process of malignant transformation. Similar to the *RAS* oncogene, the constitutive activation of *RET* and *NTRK1* leads to cell proliferation but may not be sufficient to induce malignant growth in every case [18, 19]. Recent data suggest that specific hybrid oncogenes display different malignant potential that is associated with distinct morphological phenotypes of the papillary thyroid tumors [5]. The most frequently expressed hybrid oncogene, RET/PTC1, is predominantly found in the classic PTC variant and in papillary microcarcinomas. These PTCs are usually less aggressive and account for the good prognosis of the malignancy in general [14]. On the contrary, PTCs with solid histological pattern or tall-cell variants of PTC—which are associated with aggressive behavior—predominantly express the RET/PTC3 hybrid oncoprotein also found in 80% of the tumors from the Chernobyl area [20, 21].

To summarize, the transforming capacities of the RET/PTC and *NTRK1* chimeric receptors in papillary thyroid cancers as well as the molecular background of non-rearranged PTCs remain to be elucidated. There-

TABLE 1
Synopsis of Patients and Tumor Characteristics

No.	Tissue set	Sex	Age	pTNM (1997)	pTNM (2002)	Genetics	Tissue	Histology	Follow-Up
1	4	M	49	T4a N1a M0*	T3 N1b M0	TRK-T3	lnM	G2-3	3 re-op,† (3 y, 10 mo), death t-r
2	2	F	58	T3a N0 M0	T3 N0 M0	—	nT, pCa	G1-2 (foll)	Alive (10 y, 4 mo), nrec
3	2,4	F	13	T4a N1a M0	T3 N1b M0	RET/PTC 3, TRK-x	nT, lnM	G1	Alive (10 y, 6 mo), nrec
4	3	F	72	T3a N0 M0	T3 N0 M0	—	pCa	G2 (foll)	Alive (9 y, 6 mo), nrec
5	1	M	31	T4b N1a M0	T3m N1b M0	— (FPTC)	nT, pCa	G1	1 re-op, alive (5 y, 9 mo), nrec
6	3	F	46	T4a N1b M0*	T3 N1b M0	RET/PTC 1	pCa	G2	ComT, alive (15 y, 6 mo), nrec
7	4	F	53	T2a N1a M0*	T2 N1a M0	trk	lnM	G1-2	ComT, 2 re-op,† (12 y, 2 mo), death t-r
8	4	F	65	T4a N1a M1	T4 N1a M1	—	pCa	G2	†(4 mo), death t-r
9	1	M	25	T2a N1b M0	T2 N1b M0	RET/PTC 1, TRK-x	nT, pCa	G1	1 re-op, alive (15 y, 3 mo), nrec
10	4	F	38	T4a N1a M0*	T3 N1b M0	trk	lnM	G1-2	1 re-op, alive (6 y, 2 mo), nrec
11	1	F	69	T4a N1b M1*	T3 N1b M1	—	lnM	G1	1 re-op, alive (6y), nrec
12	3	M	25	T2a N1a M0	T1m N1b M0	—	nT, pCa, lnM	G1	1 re-op, alive (14 y, 11 mo), nrec
13	2	F	29	T4a N1a M0*	T3 N1b M0	RET/PTC 1	lnM	G2-3	†(7 y, 2 mo), death t-r

Note. nT = normal thyroid tissue; pCa = primary carcinoma; lnM = lymph node metastasis; FPTC = familial papillary thyroid carcinoma; G1,2,3: tumor grading (highly, moderately, poorly differentiated cancer); foll = predominantly follicular differentiation; * = primary tumor resection performed at an outside hospital; comT = completion thyroidectomy; re-op = surgery for persistent or recurrent PTC; nrec = no recurrent or suspected tumor; t-r = tumor-related. The follow-up period from the month of primary surgical intervention up to the patient reevaluation in December 2003, or the remaining life span, respectively, is given (in parentheses).

fore, following molecular characterization of a panel of PTCs for all known *RET* and *NTRK1* hybrid oncogenes, we examined the altered gene expression in rearranged and non-rearranged tumors by a modified mRNA differential display (DD) technique. Our aim was the detection of additional or modifying molecular alterations, e.g., the identification of co-activated oncogenes and tumor-suppressor genes or the identification of genes that potentially cooperate in or modulate the process of malignant transformation following rearrangements of tyrosine kinase receptors.

PATIENTS AND METHODS

Tissue. Snap-frozen tissues (primary neoplasms and/or lymph node metastases) of 13 PTCs with or without *RET* and *NTRK1* rearrangements and corresponding normal thyroid tissues were included in the study. Informed consent was obtained from the patients according to the recommendations of the institution's Ethics Committee. Six of 13 PTCs harbored *RET* chimeras (3× *RET*/PTC1, 1× *RET*/PTC3) or *NTRK1* chimeras (2× *trk*, 1× *TRK-T3*, 2 unknown *TRK* hybrids), respectively. Two tumors expressed both types of hybrid oncogenes. Preferentially, tumors with obviously divergent histology or biological behavior (e.g., early *versus* late onset, different TNM category, tumor differentiation, and outcome) were selected. On the other hand, because the emphasis of our study was on thyroid tumor progression rather than on tumorigenesis, care was taken to mainly select malignancies with proven capability of metastasis (pN1). Four different sets of tissues derived from 13 patients (Table 1) were directly compared and examined for differentially expressed mRNA transcripts. The tissue sets were composed as follows: set 1 compares

two highly differentiated rearrangement-negative malignancies [primary carcinoma (pCa) and lymph node metastasis (lnM), respectively] with a tumor harboring both *RET*/PTC1 and *TRK-x*: patient no. 5: normal thyroid (nT) and pCa; patient no. 9: nT and pCa; patient no. 11: lnM. Patient no. 5 suffered from a rearrangement-negative PTC, but belongs to a kindred with familial papillary cancer of the thyroid gland (FPTC), and the tumor may therefore carry yet unknown FPTC-associated genetic alterations (synonymous to patient G 1.1, [22]). Tissue set 2 compares *RET*-rearranged (*RET*/PTC1 and -3, respectively) with a non-rearranged tumor of female patients: patient no. 2: nT and pCa; patient no. 3: nT and lnM; patient no. 13: lnM. Set 3 compares a tumor harboring *RET*/PTC1 with two rearrangement-negative PTCs: patient no. 4: pCa; patient no. 6: pCa; patient no. 12: nT, pCa, and lnM. Tissue set 4 compares lymph node metastases of tumors with different *NTRK1* rearrangements with a non-rearranged PTC: patient no. 1: lnM; patient no. 3: lnM; patient no. 7: lnM; patient no. 8: pCa; patient no. 10: lnM.

One-half of each tissue specimen was subjected to the preparation of nucleic acids, while the adjacent half was fixed in formalin. Following HE-staining, the diagnosis of PTC was confirmed and the amount of tumor tissue within each sample was evaluated (Fig. 2). Only PTC samples containing malignant tissue of more than 70% (pCa) or 40% (lnM), respectively, were selected for analysis.

mRNA DD. The DD analysis was refined by the use of fluorescent-labeled arbitrary oligonucleotides, electrophoresis on an automated sequencer, and a novel fragment-recovery technique using a high-performance fluorescence scanner. Our technique and its modifications are described in detail elsewhere [23, 24]. In brief, following total RNA extraction and removal of DNA contamination (Fig. 3A), reverse transcription (RT) was carried out using three different anchored oligonucleotides consisting of 15 thymidine residues plus one additional nucleotide [5'-dT(15)(A;C;G)]. The cDNA was PCR-amplified using Cy5-end-labeled oligo-dT primers and arbitrary oli-

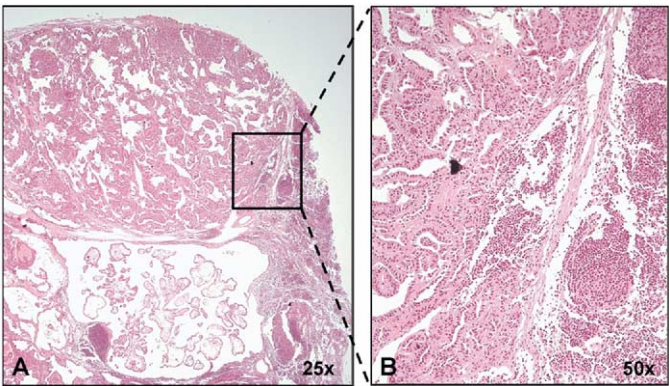


FIG. 2. (A) Tissue section (HE staining) of a fresh-frozen and paraffin re-embedded PTC lymph node metastasis exemplary for the evaluation of tumor tissue content performed before nucleic acid extraction. (B) Segmental magnification. (Color version of figure is available online.)

gonucleotides. A total of 36 different primer combinations were used. Two independent RT-PCR reactions of each RNA sample were studied simultaneously by electrophoresis using an automated sequencer. Differential expression was stated if both independent RT-PCR reactions of the same sample demonstrated equal expression levels, while the corresponding expression levels of the samples to be compared with (i.e., their amplicons of the same length) were significantly higher or lower (peak level difference > 40%).

Distinct steps within the RT-PCR protocol were time-limited, thereby allowing for only 10 reactions to be reliably and reproducibly performed at one time. Since for quality assurance independent dual PCR amplifications are carried out, the number of tissues to be compared in a single expression analysis was limited to five samples.

To recover the cDNA fragments identified by DD analysis, an aliquot of the original RT-PCR reaction was separated by conventional 6% denaturing PAGE. The gel was scanned with a fluorescence scanner and a 1:1 printout was generated that served as press copy to identify the correct spot on the gel harboring the cDNA transcript of interest. The fragments were recovered and reamplified

with the identical anchored and arbitrary oligonucleotides as used for the expression analysis. The purified amplicons were cloned into commercial vectors and the resulting plasmid DNA was sequenced to identify the differentially regulated transcripts. Of each fragment examined, at least two clones (i.e., plasmid DNA of two to five different *Escherichia coli* colonies) were sequenced to account for the fact that more than one cDNA of the same length may have been isolated from the gel slice. For sequence comparison, the subroutines BLASTN and BLASTX of the Basic Local Alignment Search Tool (NCBI-BLAST, Version 2.0) were used.

RESULTS

More than 500 band patterns were identified that represented differentially expressed transcripts (i.e., up- or down-regulated cDNA fragments of specific length) in one or more tissue samples. To limit possible false-positive results and to concentrate on the most promising candidate transcripts, fragments that demonstrated considerable expression differences and/or expression/loss of expression in only one sample were preferentially selected. A total of 276 bands were defined and the respective gel slices isolated: 69 originating from tissue set 1; 79 from tissue set 2; 71 from tissue set 3; and 57 from tissue set 4 (Table 2). The 44 most promising transcripts were selected for reamplification, which was successfully performed in 24 samples. In five cases, cloning attempts remained unsuccessful, resulting in 19 cDNA fragments inserted in vectors (Fig. 3B). Some tumors (e.g., PTCs of patients 2, 3, 6, and 9) demonstrated multiple significant different expression patterns, while others appeared quite uniform (e.g., PTCs of patients 1, 7, and 8) with

TABLE 2

Number of Transcripts Isolated by mRNA Differential Display

Patient no.	Tissue set 1	Tissue set 2	Tissue set 3	Tissue set 4
1				11 (1)
2		42 (1)		
3		21		9 (1)
4			19	
5	15 (2)			
6			31 (4)	
7				4
8				11
9	32 (4)			
10				22 (1)
11	22 (4)			
12			21	
13		16 (1)		
total	69 (10)	79 (2)	71 (4)	57 (3)

Note. The number of recovered and finally identified (in parentheses) differentially regulated cDNA fragments, respectively, is given in relation to the donating PTC patient and the tissue set examined. One isolated fragment may represent two or more different characterized gene products due to overlaying transcripts of the same base-pair length following electrophoretic separation.

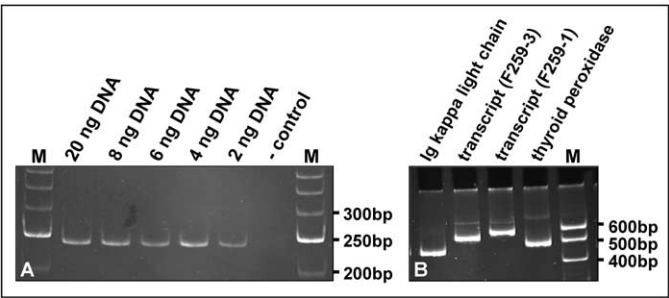


FIG. 3. (A) PAGE analysis of *RET* proto-oncogene PCR-amplification using *intronic* oligonucleotide sequences. Various amounts of genomic DNA—diluted in the same tissue sample following RNA extraction—were used as template. Down to a total of 2 ng DNA (0.1 ng/ μ l PCR reaction) was unfailingly detected, while RNA template alone did not result in detectable amplicons (negative control). Therefore, this assay was performed in every RNA isolation product to exclude even minimal contamination by genomic DNA. (B) Exemplary PAGE analysis of reamplified and cloned transcripts isolated by the refined DD technique. Subsequently, these amplification products were subjected to identification by sequencing. PAGE = polyacrylamide gel electrophoresis; DD = mRNA Differential Display; M = basepair marker.

only minor or no up-regulation compared with over-expression in the co-analyzed malignancies.

A detailed list of the genes identified by sequence comparison is given in Table 3. Three transcripts were redundantly identified, i.e., either fragments of different length coded for the same gene product or fragments of similar length encoding the same protein were isolated from different patients or different expression analyses. The following gene products were repeatedly identified: thyroid peroxidase (TPO), the 14-3-3 β /YWHAB protein, and an aberrant immunoglobulin homologous to Ig κ light chain Fab-fragment (constant region). The expression patterns of these independently isolated cDNA fragments matched in every case (Fig. 4A–C).

As expected, in strongly expressed amplification products recovered from gel slices, discrepant sequencing results can be observed when several clones of the assumed cDNA fragment were analyzed: in two cases (fragments 196 and 259), transcript-bearing plasmid DNA obtained from different *E. coli* colonies represented different gene products (Table 3, Fig. 5A and B). Similarly, more than one sequence can be found in overexpressed transcripts of seemingly the same fragment length when these fragments are recovered from different tissues samples run in the same analysis: fragments 194 (isolated from patient 9, nT) and 196 (isolated from patient 5, pCa) belong to the same expression profile displayed as bands of approximately 335 bp, but sequences representing different gene products were identified (Table 3, Fig. 5B).

Fragment 250—which was overexpressed in moderately to poorly differentiated (G2-3) PTCs when compared with highly differentiated (G1, G1-2) tumors—was identified as a gene sequence with a coding region for two completely different proteins: a V-ATPase subunit protein (ATP6V1C2) and the protein-disulfide isomerase-related protein (Table 3). The respective genes are located on complementary DNA strands on chromosome 2p25.1 in a recursive and overlapping alignment such that the partial cDNA sequence isolated by DD represents the 3' terminal region of both proteins.

Several other gene products with known or yet unknown structure and function were identified, which demonstrated differential expression in normal *versus* malignant thyroid tissue (fragment 194, 199), in lymph node metastases *versus* primary carcinoma (fragment 198), or in discriminatively differentiated tumors (fragments 26–3 etc., 63-1c, 230 etc., and 250). Among the sequenced genes, up- or down-regulation dependent on the variant of *RET* (fragments 26–3 etc, 63-1c, 259–1, and 259–3) or *NTRK1* rearrangement (fragment 26–2, Fig. 6) was observed.

DISCUSSION

Molecular-based diagnosis of solid cancers, used pre-operatively in fine needle aspiration biopsies (FNABs)

or as a tool additional to histopathological examination, relies on the identification of genetic markers or depends on the altered expression of mRNAs with specificity for malignant tissue or with association to the tumor type. Another relevant aspect of characterization of molecular markers relates to means of new therapeutic options. Patients suffering from advanced, poorly differentiated PTCs and patients with recurrent and/or metastasized PTCs that are refractory to conventional treatment (radio-iodine, retinoic acid redifferentiation etc) are in dire need of novel treatment strategies.

The approaches to characterize tumor-specific markers or differentially expressed gene products as well as the techniques applied are multifarious. This study is one of very few available gene expression analyses of PTCs using the open, nontargeted screening method of mRNA DD [25, 26]. A modified, targeted DD technique applied to thyroid cancer tissues and cell lines aimed at rapid turnover mRNAs such as cytokine and proto-oncogene transcripts [27], while another group identified mitochondrial mRNAs and proteins, respectively [28]. A number of recently published reports based on microarray techniques focused on differences between normal thyroid tissue and PTCs to identify cancer-specific gene expression profiles [29–33]. As a result, sets of genes were defined that reliably distinguish papillary thyroid cancer from normal thyroid tissue. Other studies have restricted the analysis to certain histological subtypes of papillary thyroid cancer and were able to identify genes characteristic for a subgroup of PTC with worse prognosis [34, 35]. Only one study considered the diverse genetic background of PTCs and described distinct gene expression patterns associated with the expression of the mutated oncogenes *BRAF*, *RET*, and *NTRK1* [36].

In contrast to our nontargeted “shotgun” mRNA DD method, microarray studies are usually restricted to known, short, but unique, parts of gene sequences that are attached to a commercial chip used for hybridization with cDNA. Therefore, putative yet uncharacterized gene products are scarcely identified by such techniques, whereas the DD method allows for analysis of the complete mRNA pool of a cell type, inclusive of novel transcripts. Congruously, several sequences of yet unknown putative transcripts or only recently described transcripts (KIAA1212) were identified in our study (Table 3). In addition, the mRNA DD analysis presented here is much less expensive than microarray analyses and can be performed by any well-equipped molecular biology laboratory (if a fluorescence scanner is available on the campus), while microarray studies require cost-intensive microarray facilities, commercial chips, large quantities of high-quality RNA, as well as bioinformatic expertise in handling the resulting data.

TABLE 3
Synopsis of Differentially Expressed Candidate Transcripts

Fragment	Patient no.	Expression ↑ ↓	Identification	Locus	GenBank (gi)	Function
26-2	10	mut- ↑, trk ↑ <i>versus</i> other <i>NTRK1</i> mut ↓	RP11-388G12	12p11.21	23306047	Unknown
26-3/ 63-b3/ 131-1d/ 132-3/ 188/ 189/ 221/ 223/ 265	10/ 13/ 6/ 6/ 6/ 6/ 9/ 11/ 9	RET/PTC1 ↑ ↑ <i>versus</i> mut- ↓; G2-3 ↑ <i>versus</i> G1-2 ↓; pCa > lnM; nT ↓ ↓ ↓,	Ig κ light chain (constant region)	2p12	1619959 + 21592476	Potential homologue to an aberrant immunoglobulin with transforming capacity in a nasopharyngeal cancer cell line
63-1c	13	RET/PTC1 ↑ <i>versus</i> RET/PTC3& TRK-x ↓, mut- ↓; G2-3 ↑ <i>versus</i> G1-2 ↓	Hypothetical membrane protein FLJ20533	8q13.3	34147497	Unknown
194-4	9	nT ↑ ↑ <i>versus</i> PTCs ↓	Human BAC clone CTB-161K23	7q21	1809224	Unknown
196-2	5	nT ↑ ↑, FPTC ↑ <i>versus</i> PTC3& TRK-x ↓, mut- ↓	Ras-like GTP-binding protein (RAB27A)	15q15-21.1	17432606	Exocytosis (potential involvement in secretion of thyroid hormones ?)
196-13	5	nT ↑ ↑, FPTC ↑ <i>versus</i> PTC3& TRK-x ↓, mut- ↓	KIAA1212	2p16.3	50897851	Microtubule-associated hook-related protein
198	11	lnM ↑ <i>versus</i> pCa ↓	Fibronectin type III domain containing 1 (FNDC1)	6q25	66793377	Induction of fibrillogenesis
199-2/ 200-2	11/ 5	nT ↓ ↓ <i>versus</i> PTCs ↑	14-3-3β/ YWHAB protein	20q12-13.12	31742479	Oncogene, modulates target proteins involved in cell-cycle progression, apoptosis
230-2,2/ 232-1b/ 257-b2	9/ 11/ 2	G1-2 ↑ <i>versus</i> G2-3 ↓; PTC3& TRK-x ↓ <i>versus</i> mut-(↑); nT ↑ ↑	Thyroid peroxidase (TPO)	2p25	28558981	Synthesis of thyroid hormone
250	1	G2-3 ↑ <i>versus</i> G1-2 (↓)	V-ATPase subunit protein (ATP6V1C2) and/or protein-disulfide isomerase-related protein	2p25.1	47717097	Active proton transport, pH regulation of the cell/protein scaffolding
259-1	3	PTC3&TRK-x ↑ <i>versus</i> RET/ PTC1 ↓, mut- ↓; nT ↑	Chromosome 21 segment HS21C027	21q21.2 + 4q25	7717279 + 3046280	Unknown
259-3	3	PTC3&TRK-x ↑ <i>versus</i> RET/ PTC1 ↓, mut- ↓; nT ↑	Chromosome 7 clone RP11-769P3	7q31.32	14010941	Unknown

Note. ↑/↓ = observed up- or downregulation of the respective cDNA transcript; mut +/- = tumors harboring or lacking *RET* and/or *NTRK1* rearrangements.

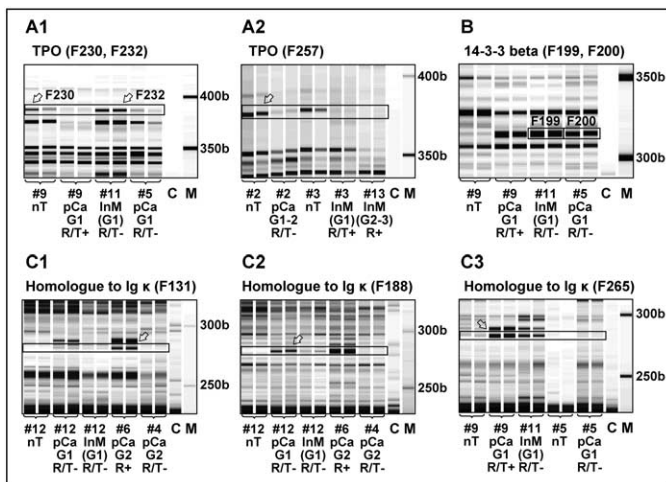


FIG. 4. Selected electrophoresis results of the differential display analysis with regard to repeatedly identified candidate transcripts. For every single expression analysis, two independent twin RT-PCR amplifications were performed and simultaneously separated. (A1–2) The expression pattern of an approximately 375b cDNA fragment (encircled bands) is depicted in two runs examining two different tissue panels. All three gene products of fragments F230, F232, and F257 were identified as thyroid peroxidase (TPO). (B) The transcript of 14-3-3 β was isolated from two strongly expressed cDNA fragments of approximately 315b found in two different malignant tumors of the same tissue panel. (C1–3) Three exemplary electrophoresis results of the most redundantly identified transcript, a sequence homologous to the constant region of Ig κ light chain, are pictured. Notably, the expression is up-regulated in primary carcinomas when compared to lymph node metastases as well as in tumors harboring *RET* rearrangements compared to rearrangement-negative malignancies. r+ = *RET* rearrangement-positive tumor; R/T+ = both *RET* and *NTRK1* rearrangement-positive tumor; R/T- = tumor without rearrangements; C = negative control, M = marker (single-stranded DNA).

To identify yet unknown molecular genetic alterations that promote or modify the malignant transformation of thyroid follicular cells or that may determine the biological behavior and clinical aggressiveness of papillary carcinomas of the thyroid gland, we used an

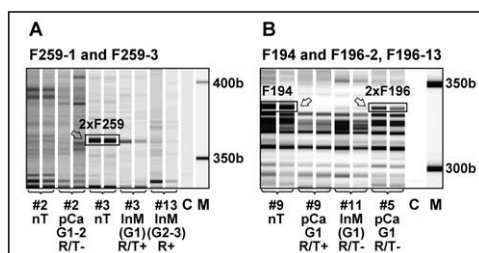


FIG. 5. Electrophoresis results of the differential display analysis with regard to bands from which more than one candidate transcript was isolated. (A) Fragment 259 revealed two different cDNA fragments of the same length, representing two genes with unknown function. (B) Similarly, two different transcript sequences were identified following recovery of the F196 band, while a third gene product was found in fragment 194 that matches the size of approximately 335b. Therefore, interpretation of these expression patterns is preliminary and awaits confirmation by quantitative expression analysis.

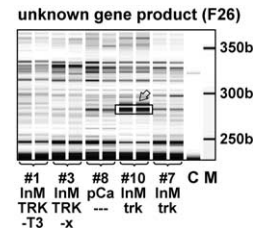


FIG. 6. Electrophoresis result depicting the expression pattern of fragment 26 which is differentially up-regulated in lymph node metastases of *trk*-bearing PTCs when compared with lymphonodal metastases of tumors with other *NTRK1* rearrangements.

open comparative expression analysis. The subgroup of papillary malignancies harboring tyrosine kinase receptor rearrangements was compared with tumors without detectable sporadic mutations of the *RET* or *NTRK1* proto-oncogene, respectively, to visualize expression profiles associated with the genetic alteration. Because the role of *BRAF* mutations in PTCs was not described when our study was initiated, the tumors were not analyzed for these mutations.

Since the experimental setting was designed to specify individual differences between *RET* and/or *NTRK1* rearranged *versus* non-rearranged tumors and to identify potentially divergent downstream alterations in solitary tumors, pooling of tissue samples (i.e., blurring of minor differences) was avoided. Three of four tissue sets studied included corresponding normal thyroid tissue and one tumor with the most common rearrangement, *RET*/PTC1. The last tissue set was specifically composed to search for differences in the expression of tumors harboring divergent *NTRK1* hybrid oncogenes. Inclusion of PTCs without detectable rearrangements of the tyrosine kinase receptors in all tissue sets additionally aimed at the identification of other transforming or modifying genes in the papillary neoplasms. The transcripts of the latter genes were expected to demonstrate differential expression in benign *versus* malignant tissues independent of the existence or absence of the aforementioned rearrangements.

The redundant expression profiles of certain transcripts (despite of utilization of different anchored oligonucleotides, completely different primer sets, or different tissue samples) as well as the almost identical band patterns of the twin pairs of independently performed RT-PCR reactions in all expression analyses confirmed the reproducibility of the laborious technique applied (Figs. 4 and 5). In this respect, the repeatedly identified up-regulation of thyroid peroxidase in benign compared with malignant thyroid tissue—with the remarkable exception of increased expression in a lymph node metastasis of a highly differentiated PTC, which seems to have maintained thyrocyte-specific features—underlines the capacity of the method to reveal individual features of the tissues examined.

Among the mRNA transcripts displaying differential gene regulation in malignant *versus* benign thyroid tissues, several gene products with known association to cell proliferation, tumor invasion and/or progression to metastasis were observed, namely 14-3-3 β protein, Rab27a, a vacuolar H(+)-ATPase subunit protein, and the protein-disulfide isomerase-related protein. Some of numerous conceivable relations of the identified mRNA expression patterns to the development and progression of papillary cancers of the thyroid gland will be described for each of the aforementioned gene products.

14-3-3 β /YWHAB

The transcript representing the 14-3-3 β protein was redundantly isolated in two different PTCs compared in the same tissue set, showing strong expression of the fragment in all carcinomas while missing in normal thyroid tissue. The identified gene belongs to a highly conserved family of at least seven isoforms of scaffold and adapter proteins, originally named for their migration positions in two-dimensional gel electrophoresis of proteins from brain extracts. 14-3-3 proteins play an important role in many processes that are relevant to cancer biology including cell-cycle progression, apoptosis, and mitogenic signaling [37]. They interact with target proteins mainly via serine/threonine phosphorylation-dependent mechanisms and modulate the stability, subcellular localization, and/or activity of their targets, e.g., by occupation of interaction domains. Modulation of proteins of the Ras downstream pathway such as phosphatidylinositol 3-kinase (PI3K) and its signaling cascade as well as interaction with pro-apoptotic proteins such as BAD (Fig. 1) have been described among the regulation mechanisms involving more than 70 identified binding partners of 14-3-3 proteins.

The 14-3-3 β gene is overexpressed in several murine tumor cell lines and its transforming capacity was shown in a rat hepatoma cell line. Transfection of the cells with antisense 14-3-3 β cDNA diminished their growth ability *in vitro* as well as *in vivo* after inoculation in nude mice [38]. Interestingly, 14-3-3 β interacts with the insulin-like growth factor I receptor (IGFIR) and thus may play an important role in the transformation pathway signaled by IGFIR [39]. This leads to the assumption that in the malignant thyroid cell IGFIR—whose expression has been demonstrated especially in thyroid tumors with aggressive clinical behavior [40]—may also be regulated by 14-3-3 β protein. Conceivably, in the thyrocyte 14-3-3 β may be an important modulating factor for other tyrosine kinase receptors such as RET and NTRK1. 14-3-3 β seems to be a new oncogene that cooperates with other oncogenes in the transformation of a cell, and our results implicate an involvement in the tumorigenesis of papillary thyroid cancers. The fact that 14-3-3 proteins

play a key role as modulators of the cell-cycle process qualifies these proteins as potential targets for anti-cancer drugs [41], which may become an important treatment option for dedifferentiated PTCs.

Rab27a

The mRNA expression of RAB27a was increased in normal thyroid tissue as well as in one highly differentiated tumor without detectable mutation (FPTC), while it was missing in other PTCs with and without tyrosine kinase rearrangements. The Rab family of proteins is part of the Ras superfamily of small GTPases and Rab proteins are considered master regulators of cellular membrane transport. Rab27a regulates the movement of melanosomes to the cell periphery of melanocytes, and it also regulates the secretion of lytic granules in cytotoxic T lymphocytes [42, 43]. The lack of Rab27a thus causes pigment anomalies and dysfunctional T lymphocytes leading to immunological defects and partial albinism, a symptom complex known as Griscelli syndrome. The decreased expression levels of Rab27a observed in PTCs may indicate the cellular dedifferentiation and the associated loss of the ability to secrete thyroid hormones.

V-ATPase C2 Subunit (ATP6C2)

The cDNA fragment identified as V-ATPase C2 subunit was overexpressed in moderately to poorly differentiated (G2-3) PTCs when compared with highly differentiated (G1, G1-2) tumors. The V-ATPase is a ubiquitously expressed membrane-bound enzyme that is composed of multiple subunits. The protein pumps protons from the cytoplasm into the lumen of vacuoles and also regulates the cytosolic pH. Because tumor cells possess high glycolytic activity and often exist in a hypoxic environment—due to accumulated acidic metabolites—maintaining of a physiological intracellular pH is essential for cellular function and survival. Hence, expression of V-ATPase has not only been observed in vacuoles but also in plasma membranes of various malignant cells. In our study, the expression level of V-ATPase mRNA correlates with the differentiation of the tumors examined. Dedifferentiated, fast-growing tumors that lack efficient blood supply are more dependent on effective mechanisms to maintain a steady pH. Therefore, overexpression of V-ATPase seems to promote cell spreading and tumor progression [44].

Protein-Disulfide Isomerase-Related Protein (hPDIR)

The above-mentioned V-ATPase subunit C2 gene partially shares its chromosomal location with the recently identified gene hPDIR, belonging to the protein-disulfide isomerase (PDI) 1 superfamily. The differentially expressed transcript isolated from one of the compared tumors was identified as a part of this over-

lapping region of the two genes that are located on opposite DNA strands. Therefore, it is not possible to pinpoint one of the two genes responsible for the over-expression in poorly differentiated PTCs at this stage of the experiment.

Protein-disulfide isomerases catalyze the oxidation, reduction, and isomerization of protein disulfide bonds and are essential for protein folding. The function of hPDIR is not fully understood: while it shares some features with PDI, it also displays several distinct functions [45]. Interestingly, the dimerization of the RET protein to a functional complex is dependent on the formation of disulfide bonds located in the extracellular domain of RET (Fig. 1). The rearranged *RET* oncogene lacks the 3' part encoding the extracellular domain, and therefore, activation of the chimeric oncoprotein may necessitate mechanisms similar to the physiological disulfide bonding.

In addition to the identification of cDNA fragments with known association to tumor development and progression, several differentially expressed transcripts representing genes with unknown function were detected:

Homologue to Ig κ Light Chain, Constant Region

Quite puzzling, the most repeatedly isolated transcript constituted of a sequence homologous to the Fab region of kappa light chain immunoglobulins. At first sight, the cDNA fragments may originate from lymphocytes present in the tumor tissue and may therefore represent an epiphenomenon in the sense of tumor-induced immune response without a role in carcinogenesis. However, the expression profile of the respective 280b fragment demonstrated consistent up-regulation in primary carcinomas when compared with lymph node metastases of the same patient or of other patients and displayed down-regulation in normal thyroid tissue. Lymphocytes self-evidently are abundant in lymphonodal tissue, while the primary carcinomas did not reveal histopathological features of round-cell infiltration that would explain the finding. Furthermore, the primary cancers displaying the highest levels of the Ig κ homologue transcript were PTCs expressing the RET/PTC1 rearrangement, while rearrangement-negative tumors showed no or significantly weaker expression. The high degree of reproducibility of the described expression pattern in different analyses of the same tissue set (Fig. C1-2) as well as the isolation of the cDNA in all four tissue panels examined (Table 3, Fig. C3) lent credence to the over-expression observed.

Therefore, the production of an aberrant immunoglobulin—carrying the constant region identified by DD—by the malignancy itself may be an alternative explanation. In accordance with this hypothesis is the reported finding of an aberrant immunoglobulin in a

nasopharyngeal cancer-derived cell line [46]. This sequence designated Tx lacked the variable regions of immunoglobulins but contained the human Ig κ light chain constant region and several binding sites for nuclear transcription factors. In addition, the gene exhibited moderate transforming activity upon transfection into murine cells. Further studies indicated that the Tx gene is not activated by point mutations but, potentially, by its interaction with other regulatory factors [46]. Another potential line of evidence is provided by the reported down-regulation of the IgG Fc binding protein (Fc γ BP) in PTCs compared with benign thyroid tissues by use of restriction-mediated differential display (RMDD) [26]. Fc γ BP binds monomeric and aggregated IgG and decreased expression may therefore facilitate immune evasion. At this point of our study, the nature of the up-regulated Ig κ homologous sequence in PTCs predominantly harboring the RET/PTC1 rearrangement remains speculative and further investigation is warranted.

To summarize, the rearrangement-specific expression profiles detected by mRNA DD may demonstrate oncogene-specific activation of downstream cascade proteins, but may also be the result of overlapping effects of modulating factors caused by additional genetic alterations. Due to the small number of tumors bearing hybrid *RET* and *NTRK1* oncogenes, we are not able to pinpoint the nature of the expression differences at this stage of the experiment. To ascertain that the altered expression patterns do have a biological correlate, every identified candidate transcript will be subjected to quantitative expression analysis using a large and representative panel of papillary cancers with and without rearrangements of tyrosine kinase receptors, including their nonmalignant counterparts of normal thyroid tissues. However, the observation of differentially regulated gene products with already known association to the development and progression of malignancies underlines the relevance of the candidate transcripts. Furthermore, our findings raise our hopes that characterization of the isolated gene sequences with unknown function will further illuminate the development of papillary thyroid cancer and may facilitate molecular diagnosis or development of therapeutic strategies.

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4. Publikationsverzeichnis

Originalpublikationen

Musholt TJ, Brehm C, Hanack J, von Wasielewski R, Musholt PB

Identification of differentially expressed genes in papillary thyroid carcinomas with and without rearrangements of the tyrosine kinase receptors RET and/or NTRK1.

J Surg Res, 2006 Mar. 131: 15-25.

Musholt TJ, Hanack J, Brehm C, von Wasielewski R, Musholt PB

Searching for Non-RET Molecular Alterations in Medullary Thyroid Carcinoma: Expression Analysis by mRNA Differential Display

World J Surg, 2005. 29: 472–482

Publizierte Vorträge

Musholt TJ, Brehm C, von Wasielewski R, Hanack J, Brauns W, Musholt PB.

Non-RET and non-NTRK1 modulating molecular factors in papillary thyroid carcinomas: expression analysis by mRNA Differential Display

22. Arbeitstagung der Chirurgischen Arbeitsgemeinschaft Endokrinologie (CAEK), Frankfurt, Dezember 2003

Abstract in: Langenbecks Arch Surg (2003); Dec, 388 (6): 436, Abstract 29

Nicht publizierte Vorträge

Musholt TJ, Hanack J, von Wasielewski R, Brehm C, Brauns W, Musholt PB.

Identifikation potentieller RET Aktivierungswege bzw. alternativer Transformationswege in medullären Schilddrüsenkarzinomen: Expressionsanalyse mittels mRNA Differential Display

21. Arbeitstagung der Chirurgischen Arbeitsgemeinschaft Endokrinologie (CAEK), Potsdam, November 2002

Musholt TJ, Brehm C, Hanack J, von Wasielewski R, Musholt PB

Identification of differentially expressed genes in PTC with and without rearrangements of the receptor tyrosine kinases RET and/or NTRK1.

Gründungsveranstaltung der European Society of Endocrine Surgeons (ESES), Pisa, Mai 2004

Musholt TJ, Hanack J, Brehm C, von Wasielewski R, Musholt PB.

Searching for non-RET molecular alterations in medullary thyroid carcinoma: expression analysis by mRNA differential display.

International Association of Endocrine Surgeons (IAES), Uppsala, Juni 2004

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Identifikation differentiell exprimierter Gene in papillären Schilddrüsenkarzinomen mit und ohne Rearrangements der Tyrosinkinase-Rezeptoren RET und/oder NTRK1
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Latein - Großes Latinum

Französisch - oberflächlich

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Hannover, 29.02.2008

Christoph Brehm

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Den Ehrenplatz am Ende der Danksagung erhalten meine liebe Frau Helena und unsere Kinder Ole und Ida. Obwohl sie meine Aufmerksamkeit in aller Regel eher von der Promotion ab- als dorthin gelenkt haben, haben sie doch einen der wichtigsten Beiträge zu deren Vollendung geleistet.

7. Erklärung nach § 2 Abs. 2 Nrn. 5 und 6 der Promotionsordnung

Ich erkläre, dass ich die an der Medizinischen Hochschule Hannover zur Promotion eingereichte Dissertation mit dem Titel „Identifikation differentiell exprimierter Gene in papillären Schilddrüsenkarzinomen mit und ohne Rearrangements der Tyrosinkinase-Rezeptoren RET und/oder NTRK1“ in der Abteilung für Allgemein-, Viszeral- und Transplantationschirurgie unter Betreuung von Herrn Prof. Dr. med. Thomas J. Musholt mit der Unterstützung durch Herrn Prof. Dr. med. J. Klempnauer ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Weiterhin versichere ich, dass ich den beantragten Titel bisher noch nicht erworben habe.

Ergebnisse der Dissertation wurden im Publikationsorgan „Journal of Surgical Research“ veröffentlicht.

Hannover, 29.02.2008

Christoph Brehm

8. Erklärung zur Straffreiheit

Hiermit erkläre ich, dass keine straf- und disziplinargerichtlichen Verurteilungen und anhängige Straf- und Disziplinarverfahren gegen mich vorliegen.

Hannover, 29.02.2008

Christoph Brehm