Detektion, Identifizierung und Charakterisierung tumorrelevanter Proteine in Serum, Gewebe und Zellen

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1 Einleitung

1.1 Proteomik – Biomarker

Im Jahr 1990 wurde das Humangenomprojekt gegründet. Ziel dieser Forschung war die Identifizierung der Basenpaarabfolge innerhalb der einzelnen Chromosomen mittels Sequenzierung. Diese Arbeit galt 2003 offiziell als erfolgreich abgeschlossen (http://www.genome.gov/10001772). Das entschlüsselte Genom bildet nun die Grundlage dafür, Erbkrankheiten und deren Mechanismen besser zu verstehen. Jedoch beruht die Wirkung der Gene überwiegend auf den von ihnen kodierten Proteinen. Somit sind die entscheidenden Informationen zum Verständnis der physiologischen und pathologischen Prozesse der Zelle in der Zusammensetzung und Funktion der Proteine verborgen [http://www.dgpf.org/dgpf-set.htm]. Kann das Genom in einem Organismus als statisch angesehen werden, so ist das Proteom als sehr dynamisch zu bezeichnen. Qualitative und quantitative Unterschiede von Proteinen werden durch Genexpression, Temperatur, Erkrankungen oder Umweltfaktoren in verschiedenen Organismen erreicht. Die Proteomik untersucht jedoch nicht nur solche Einflüsse auf das Proteom, sondern auch das Vorkommen von Isoformen, 3D Analysen, Modifikationen und Interaktionen zwischen den einzelnen Proteinen. Das Genom und Proteom stehen dabei in ständiger Wechselwirkung. Während Transkriptionsfaktoren (Martin et al., 1995) oder Polymerasen (Buratowski, 2009) an die DNA binden, um ihre Funktionalität hinsichtlich der Genexpression oder z. B. der Initiation der Transkription auszuüben, können siRNAs die Expression von Protein reprimieren (Pei et al., 2006). Bei der Expression von Proteinen entspricht die Menge der mRNA aber nicht immer der Menge der dazugehörigen Proteinen (Gygi et al., 1999). Die Halbwertszeit von Proteinen (Varshavsky, 1996), oder auch translationale Kontrollen (Schmidt et al., 2009) können diesen quantitativen Unterschied erklären. Die Tatsache, dass die Wirkung von Genen überwiegend auf den von ihnen verschlüsselten Proteinen beruht, machen das Proteom zu einem idealen Ausgangspunkt für die Suche nach tumorrelevanten Proteinen, oder auch Biomarkern.

Die Gewinnung der Ausgangsmaterialien für die Suche nach neuen Biomarkern kann sowohl durch nicht/minimal invasive als auch durch invasive Methoden erfolgen. Zu den nicht/minimal invasiven Möglichkeiten zählen unter anderem die Untersuchung des Stuhls oder Urins, der Ausstrich von Zellen des Mundraumes durch Bürstenbiopsien oder die Blutentnahme zur Gewinnung von Serum bzw. Plasma. Bei Untersuchungen des

Stuhls konnte Hardt et al. das Protein Tumor M2-Pyrovatkinase (M2-PK) als Tumormarker für Darmkrebs identifizieren (Hardt et al., 2004). Tumor M2-PK ist ein Enzym, welches nur in fötalem bzw. Tumorgewebe vorkommt, aber keinen organspezifischen Marker darstellt. Des Weiteren wird M2-PK aber auch differentiell exprimiert in Plasma von Brustkrebs- (Lüftner et al., 2000) oder Nierenzellkarzinompatienten (Weinberger et al., 2007) im Vergleich zu einer gesunden Kontrollgruppe gefunden. Urin wird im Wesentlichen für die Biomarkersuche im Urogenitaltrakt verwendet. Hierbei wurden Marker wie Bikunin für Blasenkrebs (Tsui et al., 2010) oder *kidney injury molecule-1* (KIM-1) in Nierenzellkarzinomen (Waanders et al., 2010) gefunden. Untersuchungen von Erkrankungen des Mundraumes mittels Bürstenbiopsie zeigten S100A8 und S100A9, als Dimer vorkommende Calcium bindende Proteine, in hyperproliferativen Läsionen oder Karzinomen gegenüber gesunden Kontrollen herunterreguliert (Driemel et al., 2007). Die Verwendung von Serum für die Suche nach Biomarkern hat einerseits den Vorteil, dass schnell und nich/minimal invasiv große Mengen Patientenmaterial gewonnen werden können. Andererseits besteht jedoch der Nachteil darin, dass Proteine mit großen Konzentrationsunterschieden von wenigen pmol/L bis zu hohen µmol/L (z. B. Albumin, 0,6 mmol/L) vorkommen (Service, 2003). Dabei werden oft Biomarker identifiziert, die im hohen Konzentrationsbereich liegen wie z. B., Transthyretin (Escher et al., 2007), Transferrin (Farias-Eisner et al., 2009) oder β2-Microglobulin (Hassel et al., 2004). Das 1994 erstmals beschriebene prostataspezifische Antigen (PSA) wird zwar routinemäßig als Biomarker bei Voruntersuchungen eingesetzt, hat aber auf Grund seiner geringen Sensitivität und Spezifität nur ein geringes Aussagepotential (Nogueira et al., 2009). Neben PSA zählen unter anderem auch *cancer antigen*s (CA) wie CA19.9 beim gastrointestinalen Karzinom oder CA125 beim Ovarialkarzinom zu den routinemäßig genutzten Biomarkern. Hierbei steht CA125 für das Protein Mucin16, welches sowohl beim fortgeschrittenen Eierstockkrebs, als auch bei Entzündungen im Bauchraum oder bei Schwangerschaften erhöht ist. Trotz der Nachteile beim Auffinden neuer Marker im Serum könnte Serum ein wichtiger Bestandteil bei Verlaufskontrollen eines Patienten nach einer Krebsbehandlung und anschliessender Therapie sein (Junker et al., 2009).

Die Resektion zählt zu den invasiven Methoden zur Gewinnung von Ausgangsmaterial für die Biomarkersuche. Diese Methode bietet den Vorteil, Markerkandidaten direkt im Tumorgewebe zu identifizieren, bevor diese sezerniert und somit in Körperflüssigkeiten verdünnt werden. In soliden Tumoren wie dem Kolonkarzinom oder dem

Nierenzellkarzinom konnten so S100A6 (Melle et al., 2008) bzw. CD70 (Diegmann et al., 2005) identifiziert werden.

1.2 Detektion und Identifizierung von Biomarkern

Das Auffinden von Biomarkern kann nur so erfolgreich sein, wie die vorgeschaltete Technik der Detektion. Die in den 60er Jahren entwickelten Massenspektrometer, die anfangs nur zur Massenbestimmung und Strukturaufklärung von flüchtigen Molekülen und Molekülbruchstücken benutzt wurden (Rehm, Proteinbiochemie), gelten mittlerweile zu den wichtigsten analytisch-proteomischen Methoden (Aebersold et al., 2003). Die Entwicklung hin zu Massenspektrometern, welche die Identifizierung von immer kleineren Proteinkonzentrationen in komplexen Proteingemischen ermöglicht, spielte dabei eine entscheidende Rolle. Neben der Elektrosprayionisation (ESI) (Loo et al., 1989) und dem *matrix assisted laser desorption/ionization* (MALDI) (Karas et al., 1988) zählt auch das *surface enhanced laser desorption/ionisation time of flight mass spectrometer* (SELDI-TOF-MS) (Hutchens und Yip, 1993) zu den Massenspektrometern, mit denen erfolgreich Biomarker detektiert und identifiziert werden können. Das SELDI basiert auf der MALDI Technologie, Ionenerzeugung, Ionentrennung und Ionendetektion und hat den Vorteil, dass Proteine auf verschiedene chromatographische Oberfläche, den sogenannten *ProteinChip arrays* aufgebracht werden können. Damit ist es möglich, Proteine aufgrund ihrer physikochemischen Eigenschaften selektiv an die ProteinChips zu binden und alle anderen störenden Faktoren wie Salze oder Detergenzien durch Waschen zu entfernen. Anschliessend werden die Proteine in eine energieabsorbierende Matrix (EAM) eingebettet und die Flugzeit wird wie beim MALDI in einer Vakuumröhre nach dem Prinzip des *time of flight* (TOF) ermittelt (Cotter, 1989) (Abb.1).

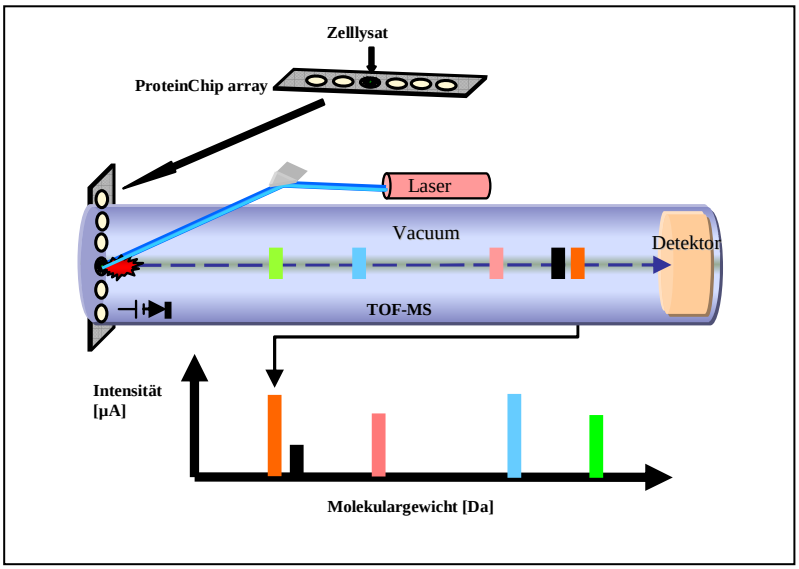

Abb.1: Schematische Darstellung der Funktionsweise der *surface enhanced laser desorption/ionisation time of flight mass spectrometry* (SELDI-TOF-MS): Proteine aus Körperflüssigkeiten wie z. B. Serum, Urin, Lungenlarvagen oder aus Zelllysaten binden entsprechend ihrer physikochemischen Eigenschaften an die verschiedenen chromatographischen Oberflächen der *ProteinChip arrays*, den sogenannten *spots*. Nach der Einbettung in eine energieabsorbierende Matrix (EAM) werden die Proteine ionisiert. Sie werden dann in einer Vakuumröhre beschleunigt und nach dem Prinzip des time of flight (TOF) vermessen. Dabei erreichen kleinere Proteine den Detektor früher als größere und erscheinen im Spektrum als Signale mit kleinerem Molekulargewicht im Vergleich zu größeren Proteinen, welche den Detektor erst später erreichen.

Eine weitere Technik, um differentiell exprimierte Proteine zu detektieren und identifizieren ist, neben der 2D Gelelektrophorese, die differentielle Gelelektrophorese (DIGE) (Tonge et al., 2001). Grundlegendes Prinzip hierbei ist die 2D Gelelektrophorese, bei der Proteine in einer ersten Dimension nach ihrem isoelektrischen Punkt und in einer zweiten Dimension nach ihrem Molekulargewicht aufgetrennt werden (Klose, 1975). Ein entscheidender Unterschied gegenüber einer herkömmlichen 2D Gelelektrophorese liegt jedoch in der Markierung der Proteine durch Fluoreszenz-Farbstoffe (z. B. Cye2, Cye3 oder Cye5). Somit ist es erstmals möglich, fluoreszensmarkierte Proteingemische von z. B. einem Patienten und einer Kontrollperson auf ein und dasselbe Gel aufzubringen, differentiell exprimierte Proteine aufgrund von Fluoreszensunterschieden zu detektieren und anschliessend mittels tryptischen Verdau zu identifizieren. Nachteil dieser Technik ist jedoch, dass für jeden Patienten und Kontrollperson mindestens drei Gele als technische Replikate benötigt werden und dies somit zeitaufwendig, materialintensiv und aufgrund der Farbstoffe sehr teuer ist. Die Auswertung der massenspektrometrisch oder

gelelektrophoretisch gewonnenen Daten erfolgt mittels biostatistischer Analysen. Die daraus resultierenden Markerkandidaten können dann nach elektrophoretischer Auftrennung im Gel tryptisch verdaut, die Proteinfragmente massenspektrometrisch vermessen und durch entsprechende Software bestimmten Proteinen zugeordnet werden.

Für die Biomarkersuche von einer größeren Probenmenge zeigt sich jedoch der Vorteil der Massenspektrometrie gegenüber der DIGE. Große Probenzahlen können mit Hilfe des SELDI in relativ kurzer Zeit auf die ProteinChips appliziert, vermessen und analysiert werden. Mit der SELDI Technologie wurden bereits eine Vielzahl von Tumorgeweben oder Körperflüssigkeiten untersucht. Der große Nachteil von Körperflüssigkeiten wie Serum (Kozak et al., 2005), Urin (Pisitkun et al., 2006; Okamoto et al., 2009) oder Cerebralflüssigkeit (Biroccio et al., 2006; Khwaja et al., 2006) liegt jedoch im Anteil der hoch konzentrierten Proteine, insbesondere beim Serum. So wurden z. B. TTR (Liu et al., 2007; Schultz et al., 2009), Apolipoproteine (Aspinall et al., 1995; Marchi et al., 2008), SAA (Cocco et al., 2009) oder Transferrin (Kozak et al., 2005) in verschiedenen Krankheiten als Biomarker postuliert. Dies zeigt, dass solche Biomarker eine relativ geringe Spezifität aufweisen und neue Techniken erforderlich sind, um neue Marker zu detektieren und identifizieren. Die Annahme, dass die Konzentration wichtiger Biomarker geringer wird, je weiter man vom Tumor entfernt im Köper danach sucht, macht eine Biomarkersuche am eigentlichen Tumor erforderlich. Hierbei ist jedoch zu beachten, dass das resektomierte Material sehr heterogen sein kann. Neben Tumorgewebe kann das entfernte Material auch Normalgewebe oder Nekrosen enthalten. Eine Biomarkersuche in solchem Gewebe führt oft zu falsch positiven Ergebnissen. Deshalb ist eine tumorspezifische Biomarkersuche nur am mikrosezierten Material möglich. Mit der Laser basierten PALM Technologie kann man Zellen (Micke et al., 2005) oder Gewebeareale (Melle et al., 2009) aus ihrem Verband lösen und proteomisch oder genomisch charakterisieren (Niyaz et al., 2005). Dies setzt jedoch eine kompetente pathologische Untersuchung des sezierten Tumorgewebes voraus, um solche falsch positiven Marker auszuschließen.

Neben den Tumormarkern, die bei einer frühzeitigen Detektion des Tumors behilflich sein sollen, sind aber auch Marker für Metastasen von enormem Interesse. Denn oft ist nicht der eigentliche Tumor, sondern die Metastasen der Grund für eine schwierige bzw. unmögliche Therapie (Narita et al., 2009). Im Falle von Kolorektalkarzinomen werden in einem von fünf Krebspatienten Metastasen diagnostiziert, welche zu einer drastischen Reduktion der Lebenserwartung führen (O'Neil et al., 2008). Dies macht neben der

Tumorbiomarkersuche eine Suche nach Metastasierungsmarkern unabdingbar. Bei Metastasierungen spielt die epitheliale mesenchymale Transition (EMT) eine entscheidende Rolle. Die Tumorzellen verlieren oder verändern ihre tumorzellspezifischen Eigenschaften und sind in der Lage, die Basalmembran zu durchdringen und z. B. in das Stroma zu migrieren, mesenchymale Eigenschaften anzunehmen, in den Blutkreislauf zu gelangen und wieder ihre epithelialen Eigenschaften durch eine mesenchymal-epithliale Transition (MET) anzunehmen (Vergara et al., 2009; Tsuji et al., 2009). Welche Faktoren eine solche Transition aktivieren und von wem sie abgegeben werden, ist noch weitgehend unbekannt. Wilkins-Port und Higgins postulierten den *transforming growth factor-beta1* (TGF beta1) und *epidermal growth factor* (EGF) als wichtige Faktoren in der Transformation von Tumorzellen und der damit einhergehenden verstärkten EMT (Wilkins-Port et al., 2007). Die meisten Studien diesbezüglich beschäftigen sich mit Tumorzellen aus der Zellkultur (Cowden Dahl et al., 2009; Graham et al., 2009). Doch durch Veränderungen der Zellen in Kultur entstehen oft realitätsferne Kultursysteme. Dies macht Studien an Tumorpräparaten, oder genauer, dem Ort der EMT, der Übergangszone zwischen Tumor und Stroma, erforderlich, um einen genaueren Blick in die Biologie des Tumors und seine Fähigkeit, Metastasen zu erzeugen, zu erhalten.

Für die proteomische Markersuche in soliden Tumoren oder Stroma ist zurzeit jedoch nur frisches oder eingefrorenes Tumormaterial zu verwenden, da dem reichhaltigen pathologischen Paraffin Material aus vielen Laboren noch kein geeignetes Verfahren zur Proteingewinnung zur Verfügung steht. Die Verbesserung solcher Verfahren zeigt jedoch erste Ansatzpunke zur erfolgreichen Separierung von Proteinen aus Paraffinmaterial (Addis et al., 2009), welcher zur Biomarkersuche verwendet werden könnten.

1.3 Charakterisierung tumorrelevanter Proteine

Neben der Identifikation von Biomarkern ist aber auch die Identifizierung neuer molekularer Mechanismen wie z. B. Proteininteraktionen innerhalb von Signalkaskaden oder Zellzyklusregulatoren ein wichtiger Ansatzpunkt im Verständnis der Tumorbiologie und somit im Kampf gegen den Krebs. Im Bereich der Regulation von Signalkaskaden konnte 1996 erstmals die Blockierung des BCR-ABL-Signalwegs gezeigt werden (Buchdunger et al., 1996), was sich bei *Koloniebildungsassay* mit Blut oder Knochenmarkzellen von CML Patienten in einer Reduktion des Wachstums von

92% - 98% widerspiegelte. Neue Erkenntnisse in der Zellzyklusregulation und somit in der Tumorbiologie könnten ähnliche Erfolge erzielen.

Um einen genaueren Einblick in die Wechselwirkungen der Proteine untereinander zu erhalten, ist es von enormem Interesse, die Vernetzungen der verschiedenen Proteinkomplexe zu erforschen. Publikationen wie z. B. die von Marais et al (Marais et al., 1996) zeigen einerseits recht einfache Signaltransduktionswege von der äußeren Zellmembran über das Zytosol bis hin zur funktionellen Wirkungsstätte der DNA im Zellkern, wohingegen andere Publikationen wahre Proteinnetzwerke mit einer Vielzahl von Verzweigungen aufzeigen, deren Komplexität zur Zeit nicht zu erfassen ist (Yamada et al., 2009). Die Identifizierung neuer Protein-Protein-Interaktionen ist daher erforderlich, um mehr über die Funktion einzelner Proteine, sowohl intra- als auch extrazellulär zu erfahren (Murzik et al., 2008) damit so ein genauerer Überblick über die biologische Funktion im Hinblick auf die Zellproliferation und generell die Tumorbiologie entstehen kann.

Die Detektion und Identifizierung von Protein-Protein-Interaktionen kann durch eine Vielzahl von Techniken erreicht werden. In den späten 80ern wurde eine Technik entwickelt, mit der es möglich war, neben den herkömmlichen Immunpräzipitationsversuchen Protein-Protein-Interaktionen durchzuführen – das Hefe-Zwei-Hybrid-System (Fields et al., 1989). Dieses neue System, welches vorzugsweise in Saccharomyces cerevisae durchgeführt wird, bedient sich oft des GAL4 Transkriptionsfaktors. Hierbei werden zwei Plasmide, welche für ein Köder, bzw. Beuteprotein kodieren, in Saccharomyces cerevisae transformiert. Das Köderprotein besteht hierbei aus einer GAL4-Bindedomäne und einer Aminosäuresequenz für die Bindung potentieller Interaktionspartner. Das Beuteprotein trägt die GAL4-Aktivierungsdomäne und einen möglichen Bindungspartner für das Köderprotein. Die Transformation beider Plasmide erfolgt in einen Hefestamm, welcher sowohl ein defektes GAL4-Gen als auch eine, den Reportergenen vorgeschaltete, Bindestelle für GAL4-Transkriptionsfaktoren besitz. Kommt es nun zur Interaktion zwischen Köderprotein und Beuteprotein, wird GAL4 funktionell rekonstituiert und eine Expression der Reportergene erfolgt. Reportergene können Basen, Aminosäuren oder auch das lacZ-Gen kodieren. Der Nachweis der Interaktion für lacZ kann dann optisch oder mittels Mangelmedium erfolgen. Der Vorteil dieser Methode liegt in den annähernd *in vivo* ähnlichen Verhältnissen, den geringen Kosten und der Robustheit von Saccharomyces cerevisae. Nachteile sind jedoch in den Modifikationen von Proteinen in der Hefe zu

sehen. Diese Modifikationen unterscheiden sich stark von denen anderer eukaryotischer Zellen und können somit zu anderen Proteinfaltungen führen. Dies wiederum führt zu falsch negativen oder falsch positiven Ergebnissen.

Eine weitere Technik zur Identifizierung von Protein-Komplexen aus Zelllysaten und anderen komplexen Proteinlösungen ist die Immunpräzipitation (IP). Hierbei ist eine Vielzahl von Varianten bekannt, die durch die spezifische Bindung der Antikörper Protein-Protein, Protein-DNA oder Protein-RNA-Komplexe aus Lösungen isolieren (Mendoza et al., 2009) Bei der Immunpräzipitation/Koimmunpräzipitation werden Antikörper gegen ein Protein an ein festes Trägermaterial gebunden, um Proteine/Proteinkomplexe aus einer Lösung zu separieren. Als Trägermaterial werden häufig Agarose oder Sepharose verwendet, an die das aus Staphylococcus aureus gewonnene ProteinA, welches die Fc-Region des Antikörpers bindet, gekoppelt wird. Der Vorteil von ProteinA Agarose ist seine enorme Bindungsmöglichkeit von Proteinen, die eine quantitativ hohe Ausbeute an einem speziellen Proteinkomplex verspricht. Jedoch kann dieser Vorteil sich auch zu einem Nachteil entwickeln, wenn nicht alle Bindungsstellen durch Antikörper oder unspezifische, zum Blockieren verwendete Proteine, besetzt sind.

Neben diesen Trägermaterialien werden auch andere wie z. B. IDM beads (Lehmann et al., 2005), oder magnetische beads (Jun et al., 2009) verwendet. Die durch Immunpräzipitation separierten Proteinkomplexe können dann in weiteren Versuchen, wie der Auftrennung in SDS-PAGE, 2D-Gelen und anschliessendem tryptischen Verdau identifiziert werden. Bei dieser Art der Präzipitation von Proteinen ist darauf zu achten, das mehrer monoklonale, bzw. ein polyklonaler Antikörper verwendet werden, da die spezifische Bindung des Antikörpers an das entsprechende Epitop mit dem Verlust von möglichen Interaktionspartners an genau dieser Stelle einhergehen kann. Der Nachteil aller Immunpräzipitationsmethoden ist jedoch die Verwendung von Antikörpern für das Separieren der Proteinkomplexe aus einer Lösung. Denn die Bindung vom Antikörper an sein Antigen besetzt immer ein Epitop, welches auch Bindungsstelle für mögliche Interaktionspartner sein könnte. Die *blue native* Polyacrylamidgelelectrophorese (BN-PAGE) umgeht diesen Nachteil, indem zur Auftrennung der Proteinkomplexe native Gele in der ersten Dimension verwendet werden Im Gegensatz zu denaturierenden Gelen, welche Detergenzien wie SDS zur negativen Ladungsgebung der Proteine verwenden, wird bei der BN-PAGE Coomassie als Ladungsträger verwendet. Das heißt, der Auftrag von Zelllysaten von Zellkompartimenten wie Mitochondrien oder Chloroplasten auf ein

natives Gel führt nicht zur chromatographischen Auftrennung von Proteinen, sondern zur Auftrennung von Proteinkomplexen nach ihrer molekularen Masse. Diese Proteinkomplexe können dann in einer denaturierenden SDS-PAGE in einzelne Proteine aufgetrennt und durch tryptischen Verdau identifiziert werden (Camacho-Carvajal et al., 2004).

1.4 Ziele der Arbeit

Biomarker sind für eine frühzeitige Erkennung von Tumorerkrankungen von enormer Bedeutung. Jedoch weisen die bisher verwendeten Biomarker wie z. B. das prostataspezifische Antigen (PSA) beim Prostatakarzinom oder *cancer antigens* (CA) wie z. B. CA19.9 bei gastrointestinalen Tumoren eine relativ geringe Sensitivität und Spezifität auf. Deshalb war das Ziel dieser Arbeit, proteomische Biomarker sowohl im Serum, Gewebe und Zellen mit großer Sensitivität und Spezifität zu detektieren, zu identifizieren und auch zu charakterisieren. Die Mikrodissektion von Tumorarealen sollte dazu verwendet werden, Marker zu detektieren, bevor diese in der Peripherie des Körpers, wie z. B. dem Serum verdünnt und kaum noch nachweisbar sind. Mögliche, so identifizierte proteomischen Marker sollen dann auf ihre Tauglichkeit hin als Serummarker getestet werden. Des Weiteren sollte ein SELDI basierendes System etabliert werden, mit dem erstmals Biomarker innerhalb eines Gewebes besser funktionalen Bereichen, dem Stroma, zugeordnet werden können. Zusätzlich sollte in dieser Arbeit neben der Charakterisierung von Biomarkern auch molekulare Mechanismen der Proliferation näher beschrieben werden, um die biologischen Prozesse in der Entwicklung hin zur entarteten Proliferation und somit hin zu Tumoren besser verstehen zu können.

2 Ergebnisse / Publikationen

Die für diese kumulative Arbeit verwendeten Veröffentlichungen zeigen Wege der Detektion, Identifizierung und Charakterisierung tumorrelevanter Proteine aus Serum, Zellen und Gewebe.

Kapitel 1 zeigt eine Möglichkeit der proteomischen Biomarkersuche in Serum von Patienten mit Mycosis fungoides (MF), einer Untergruppe des kutanen T-Zell-Lymphoms (CTCL), mittels *surface enhanced laser desorption/ionisation time of flight mass spectrometry* (SELDI-TOF-MS) auf. Dabei konnte das Protein Transthyretin (TTR) und drei seiner posttranslationalen Modifikationen stark herunterreguliert im Serum von Patienten im Vergleich zur gesunden Kontrollgruppe identifiziert werden.

Im Kapitel 2 werden die T-Lymphozyten, welche durch ihr Entarten und die Migration in die Haut das kutane T-Zell-Lymphom charakterisieren, untersucht. Nach einer bioinformatischen Analyse der massenspektrometrischen Daten zeigten die Ergebnisse eine klare Trennung der gesunden Kontrollgruppe von den CTCL Patienten. Als differentiell exprimierte Proteine konnten dabei HNP 3 identifiziert werden.

Biomarker, die vom Tumor sezerniert werden, werden in Körperflüssigkeiten verdünnt und können somit dort schlechter detektiert werden. Deshalb ist die Suche nach neuen Markern auch direkt am Tumor unerlässlich. Ein Beispiel hierfür liefert Kapitel 3, in dem Pankreaskarzinome und entsprechendes Normalgewebe mikroseziert wurden. Einer der so identifizierten Marker, HSP27 konnte anschliessend auch im Serum mittels ELISA als differentiell exprimiert zwischen Patienten und Kontrollgruppen gefunden werden. Dies wäre ohne vorherige Identifikation des Markers aus dem mikrosezierten Gewebe nicht möglich gewesen.

Um auch innerhalb eines Gewebes die Biomarker besser funktionalen Bereichen zuordnen zu können, wurde in Kapitel 4 eine räumlich hochauflösende massenspektrometrische Technik entwickelt. Mit der *tissue on chip* Massenspektrometrie (toc-MS), war es dann erstmals möglich, 100-500 stromale Zellen aus Kopf-Hals-Tumoren massenspektrometrisch zu vermessen und Tumorstroma vom Normalgewebsstroma proteomisch zu separieren.

Neben der Detektion und Identifizierung spielt die Charakterisierung tumorrelevanter Proteine eine entscheidende Rolle in der Onkologie. Damit beschäftigen sich Kapitel 5 und 6.

In Kapitel 5 wird der in Kapitel 1 gefundene Marker TTR anhand von Protein-Protein-Interaktionsstudien mittels *blue native* Polyacrylamidgelelektrophorese (BN-PAGE) charakterisiert. Dabei konnten Apolipoprotein A1, Apolipoprotein A4, Retinol bindendes Protein 4 (RBP4) und der X Rezeptor (RXR-β) als Interaktionspartner von TTR im Serum identifiziert werden. Nach dem Aufstellen der Hypothese, dass Interaktionspartner von Biomarkern ebenfalls Biomarker darstellen können, konnte Apolipoprotein A1 ebenfalls als herunterreguliert in Serum von Patienten mit CTCL gegenüber der gesunden Kontrollgruppe nachgewiesen werden.
Um die Tumorbiologie genauer zu verstehen, ist aber nicht nur die Charakterisierung von Biomarkern sondern auch molekularer Mechanismen erforderlich. Hier entschieden wir uns für den Transkriptionsfaktor E2F1, der in Zusammenhang mit DP1/2 und pRb eine entscheidende Rolle in der Proliferation der Zellen spielt. Im Kapitel 6 konnte mittels Protein-Interaktionsstudien Alien als neuer Interaktionspartner von E2F1 identifiziert werden. Funktionelle Studien zeigten eine reprimierende Wirkung von Alien auf E2F1.

2.1 Kapitel 1: Posttranslational Modifications of Transthyretin Are Serum Markers in Patients with Mycosis Fungoides

Escher N, Kaatz M, Melle C, Hipler C, Ziemer M, Driesch D, Wollina U, von Eggeling F.
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Die Studie mit dem Titel “Posttranslational Modifications of Transthyretin Are Serum Markers in Patients with Mycosis Fungoides” zeigt eine Möglichkeit, differentiell exprimierte Proteine in Serum mittels *surface enhanced laser desorption/ionisation time of flight mass spectrometry* (SELDI-TOF-MS) zu detektieren, mit XLminer biostatistisch auszuwerten und anschliessend durch 2D Gelelektrophorese zu identifizieren. Dabei konnte das Protein Transthyretin (TTR) und drei seiner posttranslationalen Modifikationen in Mycosis Fungoides (MF) gegenüber der gesunden Kontrollgruppe stark herunterreguliert gefunden werden. Validiert wurden diese Ergebnisse mittels Immundepletion und ELISA. Alle praktischen Arbeiten wurden von mir persönlich durchgeführt. Die anderen Autoren beteiligten sich durch Beratung oder Probenbereitstellung.

RESEARCH ARTICLE

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Posttranslational Modifications of Transthyretin Are Serum Markers in Patients with Mycosis Fungoides[1]

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Abstract

Cutaneous T-cell lymphomas (CTCLs) are characterized by the recruitment of malignant T-cell clones, predominantly of the CD4+ T-helper subpopulation, into the skin. Mycosis fungoides (MF) is the most common type of CTCL and accounts for almost 50% of all primary cutaneous lymphomas. The ProteinChip technology surface-enhanced laser desorption/ionization time of flight/mass spectrometry (SELDI-TOF-MS) was used to detect biomarkers in sera from MF patients ($n = 25$) and healthy controls ($n = 26$). Therefore, diluted sera were applied to IMAC30 ProteinChip arrays, and the resulting protein profiles were bioinformatically analyzed. A protein set that distinguishes MF patients from healthy controls with a sensitivity of 82.6% and a specificity of 100% was identified. Four significant peaks were identified by two-dimensional gel electrophoresis, immunodepletion, and SELDI-TOF-MS as transthyretin (TTR) and three TTR modifications. A subsequent enzyme-linked immunosorbent assay confirmed these findings. The ability to detect and identify proteins and protein modifications using SELDI-TOF-MS might reveal a better insight on this kind of disease and may lead to a better understanding and earlier detection of MF patients.

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Keywords: ProteinChip arrays, SELDI, mycosis fungoides (MF), transthyretin, transthyretin modifications.

Introduction

Primary cutaneous T-cell lymphoma (CTCL) comprises a heterogeneous group of non-Hodgkin's lymphomas involving memory T cells, predominantly of the CD4+ T-helper subpopulation, which preferentially migrate into the skin. CTCL represents the most common primary cutaneous lymphoma (65%), whereas mycosis fungoides (MF) represents the most common disease (50% of all primary cutaneous lymphomas), followed by primary cutaneous CD30+ lymphoproliferative disorders (accounting for approximately 30%, including primary cutaneous anaplastic large cell lymphoma) and the leukemic variant Sézary syndrome (with about 3%) [1]. The MF type starts mostly in middle adulthood and has an incidence of 0.4/100,000 individuals/year in the United States [2], with increasing ratio. Although treatable in its early stages, MF is frequently misdiagnosed because of its similarities to more benign forms of skin diseases. About 25% of MF patients with extensive patches or plaques will develop progressive disease. The leukemic variant Sézary syndrome is the more aggressive form, with a mean survival of 3 years from the time of diagnosis and is characterized by the presence of circulating lymphocytes of cerebriform nuclei (Sézary cells) in the peripheral blood, lymph nodes, or skin [3,4]. The etiology of MF is still unknown, but some viral infections such as human T-lymphotropic virus type I [5] or Epstein-Barr virus [6] are proposed.

To date, only a few biomarker candidates have been identified for this disease. Next to neopterin [7], β_2-microglobulin and soluble IL-2 receptor [8] have been described as possible candidate markers that are elevated in CTCL patients. The studies of Hamerlinck et al. reveal an overexpression of neopterin in the later stage of MF (except Sézary syndrome), whereas Hassel et al. described neopterin to be significantly elevated only in Sézary syndrome patients. Increased levels of neopterin [9,10], β_2-microglobulin [11,12], or soluble IL-2 receptor [13,14] can be observed in many other malignant diseases and are not specific to CTCL. Therefore, it is very important to identify biomarkers that are specific for CTCL patients and easy to detect.

It has been shown that cDNA microarrays [15] and the ProteinChip technology surface-enhanced laser desorption/ionization time of flight/mass spectrometry (SELDI-TOF-MS) [16] are appropriate tools used to distinguish between MF patients and control persons. Biomarker discovery with the SELDI-TOF-MS technique is described both in body fluids such

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as urine, blood, or serum and in microdissected tissues and cell subfractions of blood [16–18].

In previous studies, we have described our fractionation strategy using magnetic cell sorting (MACS) for $CD4^+$ and $CD4^-$ lymphocyte preparations to determine differentially expressed proteins in MF patients and healthy controls. In the course of these studies, we revealed HNP3 as a biomarker candidate for CTCL patients that separates $CD4^+$ and $CD4^-$ lymphocytes from healthy controls [16]. The aim of the current study was the detection of possible biomarkers in the sera of MF patients, using SELDI-TOF-MS, because analysis of body fluids is fast and easy to perform. For our investigation, we examined 25 MF patients *vs* 26 healthy controls using IMAC30 ProteinChip arrays. We thereby found transthyretin (TTR) and its modifications to be diminished in the sera of MF patients compared to those of healthy controls. We have also been able to detect a peak with a molecular mass of about 8590 Da in sera, which was found to be decreased in our previous fractionation studies.

Materials and Methods

Serum Samples

Twenty-five MF serum samples were obtained from the Department of Dermatology of the Friedrich-Schiller-University (Jena, Germany). The patients were between $pT_1N_0M_0$ and $pT_4pN_3cM_0$ (stages Ia–IV). As controls, we used 26 serum probes from healthy donors. All serum samples were extracted, separated, and stored (−80°C) under the same protocol before use.

ProteinChip Array

The application of IMAC30 ProteinChip array (Ciphergen, Fremont, CA) was performed according to the manufacturer's instructions. In short, 5 μl of 0.1 M Ni-sulfate was applied twice to the spots and washed away with water. Five microliters of binding buffer (0.5 M NaCl) was incubated for 5 minutes. The serum was diluted in binding buffer, and 3 μl (1.5 mg/ml) was applied to 3 μl of binding buffer on the ProteinChip. Toward incubation for 90 minutes, the spots were washed thrice with binding buffer, followed by washing with water twice. Finally, 0.5 μl of sinapinic acid was added twice, and the arrays were analyzed with a ProteinChip Reader (series 4000; Ciphergen).

Bioinformatic Analysis of ProteinChip Array Data

The resulting protein profiles between 2 and 20 kDa were subjected to CiphergenExpress Client 3.0 software and a cluster-based and rule-based data mining algorithm (XLminer 3.0; BioControl Jena GmbH, Jena, Germany). CE software was used for the processing of raw spectra and the calculation of *P* values and cluster plots according to the manufacturer's instructions. The data analysis algorithm underlying the XLminer software consists of three steps: a clustering step, a rule-extraction and rating step, and a rule-base construction step, as described elsewhere [19]. The latter two of these steps are supervised with respect to the given sample classification (CTCL *versus* unaffected). Log_2-transformed and normalized data were clustered in an unsupervised mode into two clusters ("low expressed" and "high expressed") for each peak using a modified fuzzy C-means algorithm. Using the assignment of each sample to these two states (low and high) as the condition part and the classification outcome (CTCL and unaffected) as the conclusion part, rules are generated in the rule-extraction step and rated by a statistically based rule-rating measure introduced by Kiendl and Krabs [20]. Finally, a small subset of rules from the rule list is assembled to form a rule base that can be used for the automatic classification of new patient samples. To classify a new patient sample, the cluster memberships (condition part of the rules) of all rules from the rule base that point to the same classification outcome (conclusion part of the rules) are added, and the sample is assigned to the class (classification outcome) with the highest vote.

Identification of Proteins

To identify the protein with a molecular mass of 13,746 Da that separates MF patients from healthy persons, we performed two-dimensional gel electrophoresis (2-DE) with both normal and MF sera. In short, 40 μl of serum was precipitated in 60 μl of 20% trifluoroacetic acid (TFA) and 50% acetonitrile (ACN) for 2 hours at −20°C, followed by a 30-minute step at 4°C. After centrifugation (15,000 rpm, 15 minutes, 4°C), protein pellets were washed twice in ice-cold 80% acetone. After centrifugation, the pellets were rehydrated overnight in 2% immobilized pH gradient (IPG) buffer, 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS), 0.2% dithiothreitol (DTT), 8 M urea, and 0.002% bromophenol blue. Isoelectric focusing (IEF) was carried out using 11-cm IPG strips and a PROTEAN IEF Cell (Amersham, Piscataway, NJ). The second dimension was performed using a 4% to a 12% gradient gel (Invitrogen, Carlsbad, CA) in a Novex Mini-Cell (Invitrogen). Gel staining was proceeded in Coomassie brilliant blue G-250.

Immunodepletion

About 10 μl of protein A agarose was washed in CoIP buffer containing 20 mM HEPES, 0.1 mM EDTA, and 50 mM KCl. Four microliters of anti-human prealbumin antibody (whole antiserum; Sigma Aldrich, Taufkirchen, Germany) was coupled and incubated at 4°C for 1 hour. After blocking with 3% milk powder, the agarose was washed in CoIP buffer, and 7 μl of 1:50 diluted serum from healthy donors was added. The supernatant was removed and applied to a Ni-coated IMAC30 ProteinChip array. The control with IgG antibody was treated in the same way.

TTR Enzyme-Linked Immunosorbent Assay (ELISA)

The human prealbumin ELISA kit (Assaypro, Winfield, MO) was used according to the manufacturer's instructions to detect TTR levels in sera. The serum was diluted 1:8000 in enzyme immunoassay (EIA) diluent, and 50 μl was applied to a 96-well plate and incubated for 2 hours. After washing, 50 μl of biotinylated TTR antibody was added. The supernatant was removed, the well was washed, and

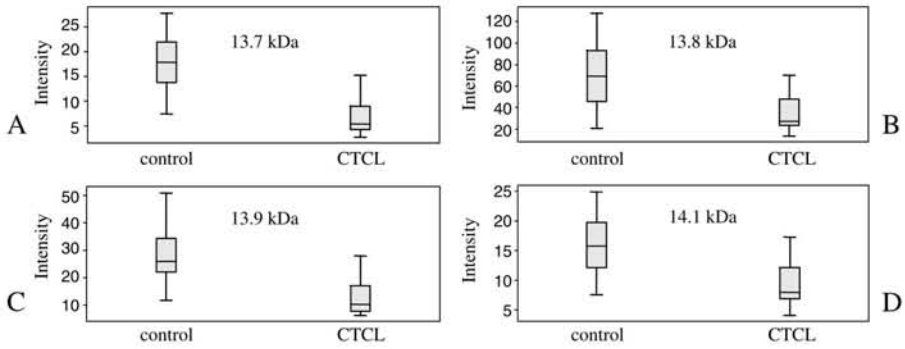

Figure 1. *Distribution of the intensity values of (A) the protein peak at 13,746 Da (identified as TTR; $P = 2.91 \times 10^{-7}$) and TTR modifications. (B) Cysteinylated form of TTR (13,878 Da). (C) CysGly-conjugated TTR (13,921 Da). (D) Glutathionylated form of TTR (14,086 Da).*

50 μl of streptavidin–peroxidase conjugate was applied. Finally, 50 μl of chromogen substrate was incubated for 10 minutes before adding 50 μl of stop solution. Absorbance was measured immediately with a UVIKON spectrophotometer (Kontron Instruments) at a wavelength of 450 nm.

Results

ProteinChip Profiling

Twenty-five MF patients and 26 unaffected control sera were analyzed with a ProteinChip Reader (series 4000; Ciphergen) on Ni-coated IMAC30 ProteinChips. After measurement and normalization, two MF and two control cases were excluded from further SELDI analyses because the intensities were too low, which means that the normalization coefficient was too high to be included in this study. Hereby, 26 signals differentially expressed with $P < .05$ in a mass range between 2 and 20 kDa were detected.

Bioinformatic Analysis

The *P* values of all detected peaks were calculated by the CiphergenExpress Client 3.0 software. The most differentiating signal between MF and unaffected sera possessed a molecular mass of 8596 Da ($P = 1.17 \times 10^{-8}$). The second specific signal with a molecular mass of 13,746 Da was identified as TTR. The distribution of intensities for both peaks comparing MF patients and controls is shown in Figures 1*A* and 2. Due to rule extraction, rating, and rule-base construction, the bioinformatic tool XL-miner (Biocontrol) revealed eight signals that, when combined, distinguish very well between MF patients and healthy controls (Table 1). This signature combination of all eight peaks revealed a sensitivity of 82.6% and a specificity of 100%.

Identification of Differentially Expressed Proteins

To identify the serum proteins differentiating between MF and healthy persons, 2-DE was performed. A number of spots in the lower molecular mass range were cut out from the gel and tryptic-digested. The peptide fingerprints of tryptic digestion generated by SELDI-TOF-MS were analyzed using the Internet database http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF. Thus, we could identify the protein TTR (P02766; www.expasy.org) with a score of 69 and a sequence coverage of 92%. This result correlated very well to one of the differentially expressed proteins found by IMAC30 Ni ProteinChip profiling (13.746 Da; $P = 2.91 \times 10^{-7}$) using SELDI-TOF-MS. This specific signal was found to be downregulated in MF sera.

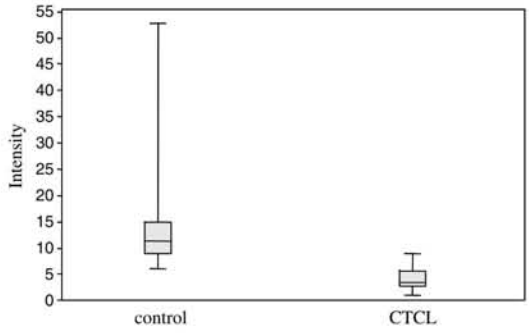

Figure 2. *Distribution of the intensity values of the protein peak at 8596 Da ($P = 1.17 \times 10^{-8}$) for MF patients and healthy controls.*

Table 1. Rule Base of the Sera Analyzed by IMAC30 Ni ProteinChips for MF Patients and Normal Controls.

Condition	Conclusion
If expression at the peak is	Then
8,596 Da high (> 3.72)	Normal
13,746 Da high (> 4.08)	Normal
13,878 Da high (> 6.18)	Normal
13,921 Da high (> 4.74)	Normal
6,664 Da high (> 6.04)	MF
8,596 Da low (< 1.54)	MF
13,878 Da low (4.54)	MF
13,921 Da low (< 3.07)	MF

All expression values are $\log_2$-transformed. The specificity of the combined rule base is 100%, and the sensitivity is 82.6% (peaks with molecular masses of 13,746, 13,878, and 13,921 Da were identified as TTR or TTR modifications).

Immunodepletion of TTR

To confirm the affiliation of TTR with the differentially expressed protein with a molecular mass of 13,746 Da, immunodepletion was performed (Figure 3). Next to the peak representing TTR, three other proteins were found depleted. According to Fung et al., two of them stand for TTR modifications. The first one, which has a molecular mass of 13,878 Da ($P = 4.80 \times 10^{-5}$), is the cysteinylated form; the second one, which has a molecular mass of 14,086 Da ($P = 2.75 \times 10^{-5}$), is the glutathionylated form. The third peak, which occurred depleted (13,921 Da), was also found to be differentially expressed ($P = 2.07 \times 10^{-6}$) when comparing MF and healthy control sera. According to Biroccio et al. [21], this signal belongs to the CysGly-conjugated form of TTR.

ELISA

To validate the differential expression of TTR, ELISA was performed on 25 MF and 26 normal serum samples according to the manufacturer's instructions. The determined median concentrations of TTR in the sera from unaffected controls were 122.98 and 46.04 μg/ml for MF patients (Figure 4). Due to Swiss Prot declaration, the normal fluctuation of TTR level in the sera was between 100 and 400 μg/ml. Receiver operating characteristic (ROC) curves were constructed for TTR serum concentrations, resulting in an area under the curve (AUC) of 0.890 (Figure 5). At a cutoff of 90.14 μg/ml, the sensitivity and the specificity were 80.8% (confidence interval = 62.1–91.5%) and 92% (confidence interval = 75–97.8%), respectively.

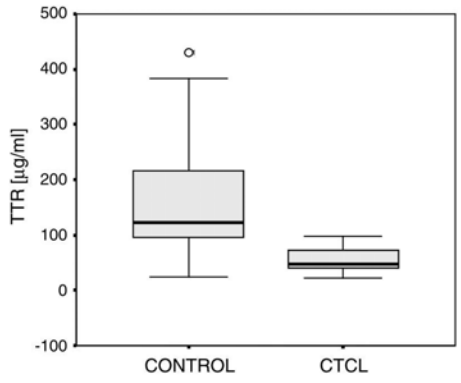

Figure 4. *Acquired TTR ELISA data are shown in a box plot. The calculated median TTR concentration is 122.98 μg/ml in unaffected controls and 46.04 μg/ml in MF patients.*

Discussion

Biomarkers are needed to facilitate the prediction of tumor progression or the early diagnosis of malignant tumors at the genomic or proteomic level. In the past years, only a few biomarker candidates, such as neopterin, soluble IL-2 receptor, or β_2-microglobulin, especially in sera, have been published for CTCLs [8,22]. However, these potential markers often lack specificity. Thus, it is important to find more significant and more specific proteins that might separate not only MF from healthy controls but also MF from the more aggressive leukemic Sézary syndrome variant.

In this study, we performed protein expression profiling using IMAC ProteinChip arrays and the SELDI-TOF-MS technique to compare 25 MF and 26 unaffected control sera. In the present study, we used 2-DE to identify potential biomarkers that might bring forward the stage at which MF is detectable. A few spots were excised from a 2-DE gel, and one of them could be identified as TTR. Immunodepletion confirmed the affiliation of TTR with the signal detected in a prior analysis using SELDI-TOF-MS. Next to TTR, three other signals were depleted. These three signals have been already described as TTR modifications: cysteinylated form

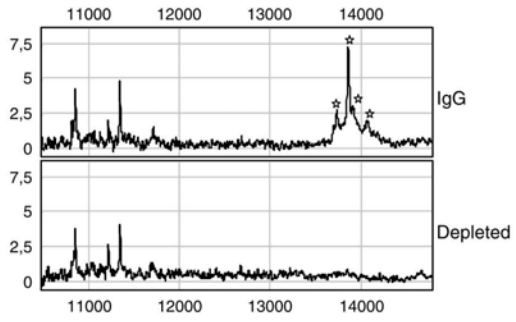

Figure 3. *Normalized ProteinChip Array profiles of the immunodepletion assay from a serum probe. To confirm the affiliation of TTR with the differentially expressed protein with a molecular mass of 13,746 Da, immunodepletion was performed. An anti-human prealbumin antibody was coupled to protein A agarose and, after serum incubation, the supernatant was loaded on an IMAC30 Ni ProteinChip. Thereby, four proteins were depleted; all of them represent TTR or TTR modifications (13.74 kDa, TTR; 13.87 kDa, cysteinylated TTR; 13.92 kDa, CysGly TTR; 14.08 kDa, glutathionylated TTR). From left to right: 13.74, 13.87, 13.92, and 14.08 kDa.*

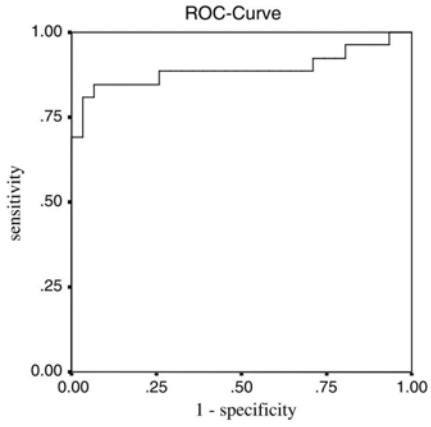

Figure 5. *The ROC curve was created, and the AUC reveals a value of 0.890. At a cutoff of 90.14 μg/ml, the sensitivity and the specificity are 80.8% (confidence interval = 62.1–91.5%) and 92% (confidence interval = 75–97.8%), respectively.*

(13,878 Da), CysGly form (13,921 Da), and glutathionylated form (14,086 Da) [21,23]. These posttranslational modifications were also found to be differentially expressed in MF patients and healthy controls, with $P = 4.80 \times 10^{-5}$, $P = 2.07 \times 10^{-6}$, and $P = 2.75 \times 10^{-5}$, respectively. The XLminer analysis of SELDI data revealed a sensitivity of 82.6% and a specificity of 100%, including the eight most differentiating peaks (Table 1). A subsequent validation with ELISA confirmed the potential of TTR to differentiate between MF and controls. The sensitivity and the specificity for the TTR as a single marker, using ELISA, were 80.8% and 92%, respectively, at a cutoff of 90.14 μg/ml. Thus, we are the first to describe TTR and its modifications as possible biomarkers for the sera of MF patients. Using this method, we have been able to detect 26 differentially expressed proteins in MF patients with $P < .05$ in a mass range between 2 and 20 kDa.

TTR is the major carrier of serum thyroxine and triiodothyronine. The transport of retinol (vitamin A) through its interaction with retinol-binding proteins is also facilitated by TTR. This liver-expressed and liver-regulated protein has also been published as a possible biomarker in other diseases such as ovarian cancer [25,27], hepatocellular carcinoma [26], and malnutrition [27].

Whereas Feng et al. and Kozak et al. only described unmodified TTR as a possible biomarker, we have been able to identify TTR itself and three TTR modifications as decreased in MF patients. Comparing our findings to the results of Kozak et al., we observed a difference in the expression of α and β hemoglobin (Hb). In contrast to the increased expression of α-Hb and β-Hb in the early stage of ovarian cancer [25], we could not detect such an overexpression of α-Hb and β-Hb. This differential result can be explained with the increased blood supply of epithelial tumors, such as ovarian or hepatocellular cancer, in contrast to lymphoma. Fung et al. examined TTR in breast, colon, ovarian, and prostate cancers compared to healthy controls. Whereas the truncated form of TTR (12.8 kDa) and unmodified TTR (13.7 kDa) were found to be significantly decreased in colon cancer, all forms of TTR (truncated, unmodified, cysteinylated, and glutathionylated) were downregulated in ovarian cancer. Comparing these findings to our results, the unmodified, cysteinylated, and glutathionylated TTR forms were also significantly downregulated in MF patients.

The results of ovarian cancer studies differed from ours on three points [24,25]. First, we could not detect the truncated form of TTR (12.8 kDa). Second, both α-Hb and β-Hb were not increased in our studies. Third, we found CysGly TTR modification to be also downregulated in MF patients. It might be possible that TTR and its modifications are specific for MF patients and may reveal a biomarker that is easy and fast to detect.

Next to TTR, we have found a signal with a 8596-Da molecular mass that was significantly decreased in MF patients. In previous studies, we have shown a protein (8565 Da) that was downregulated in MF patients on $CD4^+$ lymphocyte MACS fractionation. To date, we do not know whether it is the same protein because the identification process is still in progress.

In summary, the combination of these techniques might not be exclusively used to detect and identify differentially expressed proteins as serum biomarkers in MF patients [24,25,28]. In further studies, it might lead to the separation of the early and late stages of MF, or to the separation of MF and the more benign forms of CTCL.

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2.2 Kapitel 2: Identification of HNP3 as a tumour marker in CD4+ and CD4- lymphocytes in patients with cutaneous T-cell lymphoma

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Das kutane T-Zell-Lymphom (CTCL) ist durch das Einwandern entarteter T-Zellen des lymphatischen Systems in die Haut charakterisiert. Um von den vorangegangenen Serumuntersuchungen an die eigentliche Enstehungsquelle des Lymphoms, den CD4+ T-Zellen zu gelangen, ist eine Aufarbeitung des Patientenblutes erforderlich, um neue Biomarker am Entstehungsort von CTCL zu detektieren und zu identifizieren.

Mittels Biocoll Dichtegradienten wurden die Lymphozyten separiert und mittels *magnetic cell sorting* (MACS) in CD4+ and CD4- Zellen fraktioniert. Nach massenspektrometrischer Vermessung der isolierten Proteine konnte die MF von der Kontroll CD4- Gruppe mit einer Sensitivität und Spezifität von 96%, wohingegen MF vs. Kontrolle CD4+ mit einer Sensitivität und Spezifität von 91,7% bzw. 84% separiert werden. Anschliessend konnte HNP3 als differentiell exprimiertes Protein identifiziert werden.

Bärbel Spies-Weisshart, Annett Bleul und ich waren an der Fraktionierung und massenspektrometrischen Vermessung der Lymphozyten beteiligt. Alle anderen Arbeiten wurden von mir persönlich durchgeführt.

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Identification of HNP3 as a tumour marker in CD4+ and CD4– lymphocytes of patients with cutaneous T-cell lymphoma

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ABSTRACT

Cutaneous T-cell lymphomas (CTCL) are characterized by malignant proliferation of skin homing T-cells. Although prognosis is generally good, reliable markers are needed to identify patients at risk for a more aggressive course. ProteinChip (SELDI) technology was used as a tool for the discovery of protein patterns in lymphocytes from patients with CTCL ($n = 25$) and unaffected controls ($n = 25$). Lymphocytes were separated in CD4+ and CD4– fractions by magnetic cell sorting (MACS). Each whole protein extract was analysed by ProteinChip technology. The resulting protein profiles were submitted for bioinformatic analysis including a clustering algorithm, a rule extraction, a rating and a rule-base construction step. For the generated combined rule base for the CD4– cell fraction, both the sensitivity and specificity for the prediction of CTCL reached 96%, while for the CD4+ fraction they were 92% and 84%, respectively, for sensitivity and specificity. The most significant peak at 3489 Da could be identified as HNP3, an α-defensin, by immunocapturing. These results open up both the possibility for the use of this protein signature, especially HNP3, to more effectively monitor and screen CTCL, and the avenue to identify the other relevant peaks for a better understanding of the development of this tumour.

1. Introduction

Malignant lymphomas can affect the integument primarily and secondarily. If no extracutaneous manifestations can be detected with routine staging methods, a primary cutaneous lymphoma can be assumed. The special status of cutaneous lymphomas results from the skin associated control circuit of lymphocytes that recycle between skin and lymph node. The majority of cutaneous lymphomas are classified as T-cell lymphomas. More than 90% of the T-cell lymphomas are represented by mycosis fungoides (MF) and the leukaemic variant known as Sézary syndrome (SS). Cutaneous T-cell lymphomas (CTCL) of MF type start mostly in middle adulthood and have an incidence of 0.4/100,000 individuals/year in the US [1] but this is on the increase. CTCL is classified as a peripheral T-cell lymphoma of low malignancy with a

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prolonged indolent course. Prognosis depends mainly on the stage and severity of skin involvement and lymph node status. At the beginning of the disease histological diagnosis can be especially difficult. While life expectancy in patients with skin involvement T1 is not reduced at all, the 10-year survival rate in patients with T3 and T4 is as low as 40%. At this time, clear cut prognosis for an individual case is not possible [2].

Despite enormous efforts only a few tumour disease relevant markers have been established that can be used for early diagnosis or for a better therapy in malignancies [3]. This is despite the fact that new high parallel genomic and proteomic techniques have been established in the last few years. Up to now, there is also no highly specific tumour marker for CTCL although an increase of lactate dehydrogenase (LDH) is associated with a worse prognosis for the patient in tumour stage or erythrodermia [4]. Although numerous potential candidates have been investigated, none are yet used routinely in clinical settings. One candidate marker is neopterin, which are pyrazoino-pyrimidine molecules that are produced after IFN-γ stimulation and indicate activation of the cellular immune response [5]. Neopterins were elevated in patients with higher stages of CTCL's compared to patients with psoriasis and atopic eczema, but not in patients with SS where they were only slightly elevated. Increased neopterin levels are seen in many other malignant diseases and are not specific to CTCL. Another candidate is the α chain of soluble IL-2R (sIL-2R). Its concentration was found to be increased in advanced tumour stages of CTCL. The correlation between sIL-2R and the severity of the skin and lymph node status was better for sIL-2R than for LDH or the β2-microglobulin [6].

For the discovery of new biomarkers at the proteomic level, surface enhanced laser desorption/ionization-mass spectrometry (SELDI-MS)-based ProteinChip technology is one of the most promising techniques [7]. This technology makes use of affinity surfaces to retain proteins based on their physico-chemical characteristics, followed by direct analysis by time of flight mass spectrometry (TOF-MS) [8]. Thus, proteins being retained on chromatographic surfaces can be easily purified from contaminants such as buffer salts or detergents, thus eliminating the need for pre-separation techniques, as required with other MS techniques. Furthermore, the low sample requirements of this technique are ideal for small biopsies, microdissected tissue or cell subfractions of blood [9].

Until now, biomarker discovery with the ProteinChip technology was mostly done by analyzing body fluids like serum or urine, as body fluid analyses are fast and easy to perform by direct application on the arrays. Nevertheless, it is known that intra-individual changes in serum are high. Hence, biomarkers responsible for the genesis and progression of cancer must be present at a high level to be observed above normal changes [10]. CTCL blood samples fractionated by FACS or MACS open up the opportunity to analyze those cells that are most likely involved in tumour genesis and progression.

When specific alterations between the protein profiles are detected by ProteinChip technology, single peaks can be isolated and identified by either 2-DE or ProteinChip technology [11] and by collision-induced dissociation (CID) using a ProteinChip interface coupled to a tandem mass spectrometer [12].

In the study presented here, fractions of CD4+ and CD4− lymphocytes from 25 CTCL patients and 25 normal controls were analysed on ProteinChip Arrays, because it is known that CD4 positive lymphocytes are activated in CTCL. The resulting protein profiles were submitted to a clustering algorithm, a rule extraction and rule base construction step which together excluded the possibility of finding a protein pattern by chance. For the prediction of CTCLs, the generated combined rule base resulted in a specificity of up to 84% and sensitivity of 92% for the CD4+ cell fraction and 96% specificity and sensitivity for the CD4− fraction.

2. Materials and methods

2.1. Processing of blood samples

All blood samples of CTCL patients (n = 34) were obtained from the Clinic of Dermatology of the Friedrich-Schiller-University Jena or the Clinic of Dermatology in Dresden, Germany with informed consent. CTCL diagnostics were performed in accordance with the guidelines of the German Cancer Society (AMWF) and were based upon clinical investigations: ultrasound investigations of lymph nodes, spleen and liver; routine laboratory investigations including: a complete differential blood count, Sezary cell count, liver enzymes, lactat dehydrogenase, renal parameters and inflammatory parameters (Westergreen blood sedimentation rate, C-reactive protein); and histology of lesional skin, lymphnodes (when enlarged) and bone marrow (when indicated). In addition to hematoxylin-eosin stains, immunostains and PCR for T-cell receptor were performed. CTCL were classified according to EORTC recommendations. Classification of all evaluable patients, who were all active, are given in Table 1. Blood samples from healthy sex-matched donors were used as a control (n = 30). These normal samples (5 ml) were stimulated with phytohaemagglutinin (PHA) to activate lymphocytes. Blood samples were further processed not longer than 12 h after sampling.

2.2. Separation of lymphocytes by MACS

Ten ml of heparinized blood was quartered in aliquots of 2.5 ml, diluted with 2.5 ml phosphate buffered saline (PBS), layered onto 5 ml chilled Biocoll (density 1.077 g/ml, Biochrome AG, Germany) and centrifuged at 800g for 30 min (without brake) at room temperature. After this density gradient separation, the resultant opaque interface containing lymphocytes was carefully transferred with a syringe into a centrifuge tube and washed two times with PBS.

The washed cells were resuspended in 80 μl of PBS and stained with CD4 microbeads (Miltenyi) for 15 at 4 °C. The PBS washed cells were centrifuged at 600g for 10 min, washed and resuspended in 500 μl PBS-FCS. Afterwards the cells were separated on a miniMACS column which was placed in a MACS magnetic field (Miltenyi Biotec GmbH, Germany) according to the manufacturer's instructions. The non-retained cells containing all other lymphocytes except CD4+ is further referred to as the CD4− fraction. The magnetically retained cells were eluted after washing and are designated as the CD4+ cell fraction. Before and after MACS, the cell number was analysed with a Neubauer counting chamber.

Table 1 – Individual staging of evaluable patients and their classification according to EORTC

Patient No.	TNM	EORTC
165	pT2cN0cM0B0	Mycosis fungoides
167	pT4pN3cM0B0	Mycocis fungoides
168	pT3cN0M0B0	Mycosis fungoides
169	pT3cN0cM0B0	Large cell CTCL, CD30-positive
171	pT2cN0cM0B0	Mycosis fungoides
172	pT2cN0cM0B0	Mycosis fungoides
173	pT2N0M0	Mycosis fungoides
174	pT3pN1pM1B0	Mycosis fungoides
175	pT3pN1cM0B0	Mycosis fungoides
176	pT3cN0cM0B0	Mycosis fungoides
177	pT3pN3cM0B0	Mycosis fungoides
178	pT2cN0cM0B0	Mycosis fungoides
181	pT2cN0cM0B0	Mycosis fungoides
182	pT3pN1cM0B0	Mycosis fungoides
184	pT4pN1cM0B0	Mycosis fungoides, erythrodermatic variant
185	pT2cN0cM0B0	Mycosis fungoides
186	pT3cN0cM0B0	Mycosis fungoides
187	pT2cN0cM0B0	Mycosis fungoides
188	pT1cN0cM0B0	Mycosis fungoides
190	pT2cN0cM0B0	Mycosis fungoides
194	pT2cN0cM0B0	Mycosis fungoides
195	pT1cN0cM0B0	Mycosis fungoides
196	pT2cN0cM0B0	Mycosis fungoides
197	pT2cN0cM0B0	Mycosis fungoides
198	pT3N1M0	Mycosis fungoides

2.3. *Profiling of CD4+ and CD4− fractions*

The protein lysates from CD4+ and CD4− lymphocyte fractions were analysed on a hydrophobic reverse phase array (H4; Ciphergen Biosystems Inc., Fremont, CA) as described elsewhere [13]. In brief, array spots were preincubated with 5 μl acetonitrile and rinsed with deionised water. Then 2 μl of sample extract were spotted on ProteinChip Arrays and allowed to dry. After washing three times with the binding buffer, two times 0.5 μl sinapinic acid (saturated solution in 0.5% TFA/50% acetonitrile) was added and mass analysis was performed in a ProteinChip Reader (PBS-II, Ciphergen Biosystems Inc., Fremont, CA) according to an automated data collection protocol.

2.4. *Bioinformatic analysis of ProteinChip Array data*

The resulting protein profiles between 2 and 20 kDa were subjected to CiphergenExpress (CE) 3.0 software and a cluster and rule-based data mining algorithm (XL-Miner 3.0, BioControl). The CE software was used for the processing of raw spectra and the calculation of *P*-value and cluster plots according to the manufacture's instruction. The data analysis process with XL-Miner consists of a clustering step, a rule extraction and rating step, and a rule-base construction step as described elsewhere [14]. All these steps are supervised with respect to the given sample classification (CTCL vs. unaffected). Log 2-transformed and normalized data were clustered in a supervised mode into two clusters – "low expressed" and "high expressed" – for each peak using a modified fuzzy c-means algorithm [15]. In rule-extraction rules are generated and rated by a statistically based rule rating measure introduced by Kiendl and Krabs [16]. Finally, a small subset of rules from the rule list can forms a rule base that can be used for the automatic classification of new patient samples. To classify a new patient sample, the cluster memberships (condition part of the rules) of all rules from the rule base that point to the same classification outcome (conclusion part of the rules) are added and the sample is assigned to the class with the highest sum.

2.5. *Identification of differentially expressed protein peaks*

For immunocapturing, 3 μl (60 ng) of anti-human monoclonal antibody for α-defensin 1–3 (HNP1–3; T-1034; BMA Biomedicals; Augst, Switzerland) were incubated with 10 μl protein A-agarose (Sigma) for 15 min on ice. The sample was centrifuged and the supernatant was discarded. Thereafter, the resulting sediment was incubated with blocking solution containing 2% milk powder for 30 min on ice. The supernatant was discarded and the pellet was washed three times with a buffer (COIPB) containing 20 mM Hepes (pH 7.8), 50 mM KCl, 0.1 mM EDTA and 0.05% CHAPS. Afterwards, 5 μl of a lysate from CTCL CD4− cells were incubated with this pellet for 1 h on ice. As a negative control an unspecific antibody (IgG rabbit) was coupled to the protein A-agarose and treated in the same way. After incubation, samples were washed three times with COIPB. The proteins were eluated in 12 μl elution buffer and 3 μl of supernatant were analysed on SAX2 ProteinChip Array.

3. Results

3.1. *Separation of lymphocytes by MACS*

The density centrifugation resulted in 10^5–10^7 lymphocytes. The resulting cell numbers were strongly dependent on the time between blood collection and the further processing.

After MACS, the cell numbers for CD4+ and CD4− cells was between 10^4 and 10^5.

3.2. *Profiling of CD4+ and CD4− fractions*

For this study, sample of both CD4+ and CD4− fraction from CTCL and normal controls were applied to H4 arrays and analysed on a PBS-II system. In the low range (2–20 kDa) up to 155 peaks were detected with normalized intensities with a S/N of 3. Spectra with corresponding to low peaks were excluded, so that 25 patients samples (Table 1) and 25 control samples were further analysed. After evaluation, CiphergenExpress software data were exported in a csv-file format for bioinformatic processing.

3.3. *Bioinformatic analysis*

The *P*-values of all detected peaks were calculated by the CiphergenExpress software. For the CD4− fraction the best value (3.05×10^{-7}) was found for the peak at 3490 Da and for the CD4+ lymphocytes at 8565 Da (1.7×10^{-6}). Distribution of intensities for these peaks for controls and patients with CTCL are shown in Figs. 1 and 2.

The cluster and rule based data mining method (XL-miner) described above revealed six peaks for the CD4− fraction (Fig. 3, Table 2) whose combination is relevant to distinguish between unaffected or patients with CTCL and five such peaks for the CD4+ fraction (Fig. 4, Table 3). After combining the generated rules, the calculated sensitivity and specificity was 96% for the CD4− fraction. For the CD4+ lymphocytes a sensitivity of 91.7% and a specificity of 84% was revealed.

3.4. *Identification of HNP3*

To confirm that HNP3 are matching to the differentially expressed peak at 3489 Da identified by ProteinChip analysis an immunoassay was performed using monoclonal antibodies against HNP1–3. In this procedure, a specific anti-HNP1–3 antibody bound on protein A-agarose captured α-defensin 1–3 from the CTCL patients' CD4− cell fractions. The captured proteins were eluted from the beads and applied to an SAX2 ProteinChip for analysis by SELDI-MS. The spectra of the analysis showed peaks corresponding to HNP1–3. In a control assay using protein A-agarose beads without a specific antibody no proteins specific for HNP1–3 was captured (Fig. 5).

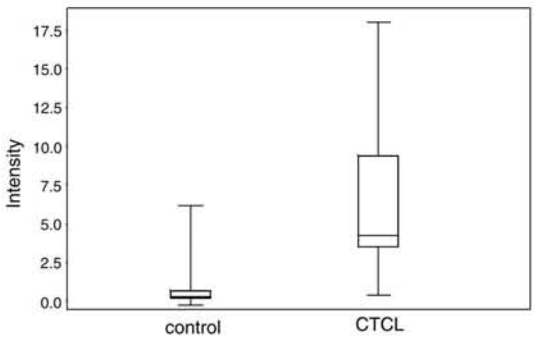

Fig. 1 – Distribution of intensity values of the protein peak at 3489 Da (later identified as HNP3) for the CD4− fraction from patients and normal controls ($P = 3.05 \times 10^{-7}$).

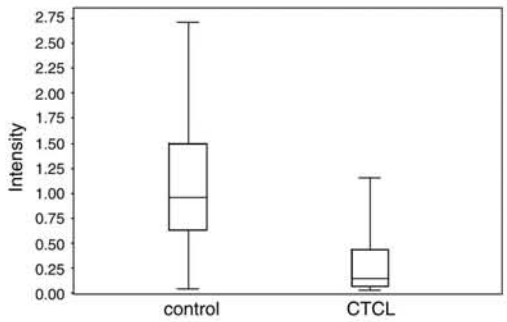

Fig. 2 – Distribution of intensity values of the protein peak at 8565 Da for the CD4+ fraction from patients and normal controls ($P = 1.7 \times 10^{-6}$).

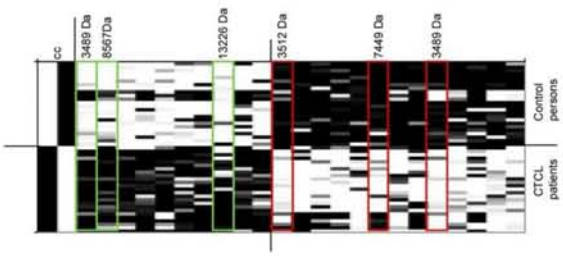

Fig. 3 – Rule list (α = 95) with relevant rules (see Table 2) for the prediction of CTCL (red frame) and normal controls (green frame) of the CD4− cell fraction. Samples are clustered horizontally, peaks vertically. The specificity of the combined rule base is 95.8%, the sensitivity 96%. The peak with 3489 Da was identified as HNP3. cc: clinical classification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

New biomarkers or biomarker patterns found by genomic or proteomic high-through put techniques should enable scientists and medical staff to make more reliable early diagnoses of certain human diseases, especially malignant tumours, and to facilitate the prediction of their progression. In this way, biomarkers could contribute to a more differentiated, individually orientated tumour therapy. Despite enormous efforts only a few relevant markers have been presently established for tumour diseases [3]. For CTCL it would be desirable to have a serum parameter that could be used for diagnosis and, more importantly, enable clinicians to monitor the course of the disease during therapy.

One of the most promising proteomic tools for the detection of new proteomic cancer biomarkers is Ciphergen's ProteinChip technology [10]. This technique has been

Table 2 – Rule base for the CD4− cell fraction to distinguish between CTCL patients and normal controls

Condition (IF)	Conclusion (THEN)
Expression at peak 3489 Da high (>2.07)	CTCL
Expression at peak 3512 Da high (>0.52)	CTCL
Expression at peak 7449 Da high (>1.76)	CTCL
Expression at peak 3489 Da low (<1.39)	Normal
Expression at peak 8567 Da high (>0.09)	Normal
Expression at peak 113,226 Da low (<3.96)	Normal

All expression values are log-2 transformed. The specificity of the combined rule base is 95.8%, the sensitivity 96% (3489 Da was identified as HNP3).

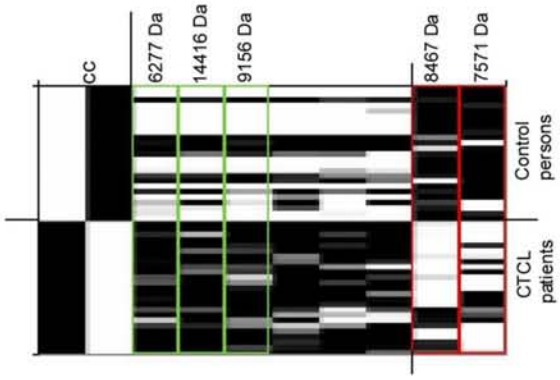

Fig. 4 – Rule list (α = 95) with relevant rules (see Table 3) for the prediction of CTCL (red frame) and normal controls (green frame) of the CD+ cell fraction. Samples are clustered horizontally, peaks vertically. The specificity of the combined rule base is 84%, the sensitivity 91.7%. cc: clinical classification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

predominantly used for body fluids, as they are fast and easy to analyse by direct application on the ProteinChip Arrays. Despite the relatively high inter- and intra-individual changes in serum, a large number of studies using body fluids as starting material have been published on serum or other body fluids [17]. This pursuit for markers is motivated by the realization that if they can be found through bioinformatics processing they would be ideal for screening high-risk individuals or even individuals without elevated risk, as discussed by Kozak and co-workers in a study of ovarian cancer [18] and others as well.

Table 3 – Rule base for the CD4+/− cell fraction to distinguish between CTCL patients and normal controls

Condition (IF)	Conclusion (THEN)
Expression at peak 7571 Da high (>−2.52)	CTCL
Expression at peak 8565 Da low (<−2.72)	CTCL
Expression at peak 6277 Da high (>0.51)	Normal
Expression at peak 9156 Da high (>−1.95)	Normal
Expression at peak 14,416 Da high (>−3.17)	Normal

All expression values are log-2 transformed. The specificity of the combined rule base is 84% and sensitivity 91.7%.

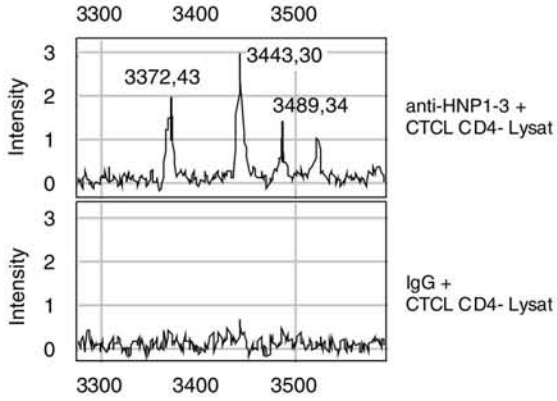

Fig. 5 – Normalized ProteinChip Arrays profiles of the immunocaptured assays of CTCL CD4− cell lysate. For HNP1–3 identification, CTCL a CD4− cell lysate was used as the starting material for immunocapturing assays using the corresponding monoclonal antibody bound on protein A-agarose. The peaks at 3.37, 3.44, and 3.48 representing HNP1–3 were clearly detectable in samples eluted from the protein A-agarose. In control assays with an unspecific antibody no HNP1–3 were captured.

In contrast to serum, the analysis of tissues or fractionated cells is more time consuming as microdissection or cell sorting is necessary. However, the chance to find a reliable tumour marker might be higher than in serum. At the very least, there is higher probability to obtain more information about the biological mechanisms leading to the genesis and progression of cancer [19,20].

Diseases like Mycosis fungoides and Sezary syndrome have already been investigated with the technique of cDNA-Array [21]. Herein, we have demonstrated that the method of MS can be applied to distinguish at the protein level between CTCL patients and control persons.

Intriguingly, no studies analyzing blood fractions have been reported in the analysis of these tumours, although this seems a more promising way using either tissue or even total serum. Especially in CTCL this seems the best choice to gain T-lymphocytes that could not easily be isolated from skin.

In our study, we therefore did not analyse serum, but nucleated cells from blood samples. As a separating method we used magnetic bead-coupled antibody against CD4, because it is known that CD4 positive lymphocytes are activated in CTCL. As control, we have therefore used PHA-activation (to stimulate lymphocytes) of the control samples to compare CTCL patients and control persons. In this way, we achieved isolation of a CD4 positive fraction containing T-lymphocytes and a CD4 negative fraction containing all other nucleated cells (e.g., granulocytes). Data from the ProteinChip analysis were submitted to bioinformatic tools to obtain a classifier or a signature to differentiate between patients and normal controls. The results gave rise to a signature of five to six proteins, which could classify the two groups (CTCL CD4− vs.

PHA activated CD4− controls) up to 96%. This is, to the best of our knowledge, the most suitable marker for CTCL so far. Interestingly, the CD4− fraction revealed the most significant results. This might be due to the extraordinary good predictor at 3489 Da.

In a former study, we had shown that colorectal cancer include HNP1–3 [22]. The protein pattern also generated using ProteinChip Technology SELDI TOF MS was identical in this mass range to the protein pattern derived from CTCL. According to this information, we investigated the patients' material for HNP3 expression and precipitated a protein with a mass of 3489 Da with an monoclonal antibody against HNP1–3.

This peak, identified as HNP3 belongs to defensins, which are small antimicrobial peptides that contain six cystein residues which form three disulfide bonds. On the basis of the position of these residues α- and β-defensins have been classified. So far six α-defensins have been identified in humans (human neutrophil proteins 1–3 [HNP1–3] and human defensins 5 and 6 [HD-5 and -6]), but many more probably exist [23]. HNP1–3 may also be expressed in intestinal epithelial cells under certain conditions. In a recent study, expression of HNP1–3 was observed in epithelial cells of the ileum and colon in cases of inflammatory diseases but not in normal intestinal tissue [24]. Whether this reflects the induction of gene expression or the uptake by epithelial cells of peptides released by neutrophils in the vicinity remains to be determined [25]. In different cancer entities like renal cell carcinoma (RCC) [26] or oral squamous cell carcinoma (OSCC) [27], HNP1–3 were also found to be up-regulated. Defensins may play a further role in adaptive immunity given that HNP1 and -2 are chemotactic for human T cells both in vitro and in vivo [28]. Both α- and β-defensins have the capacity to chemo-attract immature dendritic cells [29]. Overall, defensins seem to play, besides their antimicrobial function, an important and up to now unclear role in immunity and in progression of tumours [30]. The behaviour of α-defensins might be also crucial in CTLC, but further investigations have to be performed to obtain deeper insights into their particular role in lymphoma. So far, this is the first study to demonstrate the presence of human α-defensins 1–3 in CTCL patients. The identification of the other relevant proteins found in the signature for CD4+ and CD4− fraction is in progress and might contribute to our further knowledge on the biology of CTCL.

It is possible that the signature detected was generated by chance considering the ratio of features to patients. However, this is unlikely given that the two bioinformatic tools we employed used conservative algorithms designed to avoid finding significance in random associations. Still, further analysis must be performed and it should also be investigated whether or not the protein signature we found, especially HNP3, is present in patient serum, as this would greatly facilitate adoption of this marker in clinical use. In conclusion, we have established a promising procedure combining ProteinChip technology and bioinformatic tools that allows a screening for CTCL with high sensitivity and specificity. The specific proteomic signatures described here, even without the knowledge of all respective proteins, can serve as an additional diagnostic parameter for a better insight into the genesis and progression of CTCL.

Conflict of interest statement

None declared.

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2.3 Kapitel 3: Protein Profiling of Microdissected Pancreas Carcinoma and Identification of HSP27 as a Potential Serum Marker

Melle C, Ernst G, Escher N, Hartmann D, Schimmel B, Bleul A, Thieme H, Kaufmann R, Felix K, Friess HM, Settmacher U, Hommann M, Richter KK, Daffner W, Täubig H, Manger T, Claussen U, von Eggeling F.
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Diese Veröffentlichung ist bei der Biomarkersuche im Gewebe einzuordnen. Bei diesen Untersuchungen wurden Hals-Kopf-Tumore verwendet, um neue Marker zu detektieren und zu identifizieren. Dabei wurden Proteine aus mikroseziertem Material von Tumorgewebe mit Normalgewebe massenspektrometrisch verglichen. Biostatistische Analysen ergaben eine klare Trennung beider Gruppen wobei HSP 27 und DJ-1 als differentiell exprimiert identifiziert wurden. Diese Ergebnisse wurden mittels Immunhistochemie (IHC) bestätigt. Die weiteren Untersuchungen an Serum der Kopf-Hals-Tumorpatienten mittels *enzyme linked immunosorbent assay* (ELISA) ergaben ebenfalls eine Heraufregulation von HSP 27 im Vergleich zur Kontrollgruppe. Die Ergebnisse der Gewebedaten wurden hauptsächlich durch Christian Melle in Kooperation mit Herrn Ernst, Frau Bleul und mir erzielt. Die biostatistischen Analysen und die Serumuntersuchungen führte ich durch.

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Proteomics and
Protein Markers

Protein Profiling of Microdissected Pancreas Carcinoma and Identification of HSP27 as a Potential Serum Marker

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Background: **Patients with pancreatic adenocarcinomas have a poor prognosis because of late clinical manifestation and the tumor's aggressive nature. We used proteomic techniques to search for markers of pancreatic carcinoma.**

Methods: **We performed protein profiling of microdissected cryostat sections of 9 pancreatic adenocarcinomas and 10 healthy pancreatic tissue samples using ProteinChip technology (surface-enhanced laser desorption/ionization). We identified proteins by use of 2-dimensional gel electrophoresis, peptide fingerprint mapping, and immunodepletion and used immunohistochemistry for in situ localization of the proteins found. We used ELISA to quantify these proteins in preoperative serum samples from 35 patients with pancreatic cancer and 37 healthy individuals.**

Results: **From among the differentially expressed signals that were detected by ProteinChip technology, we identified 2 proteins, DJ-1 and heat shock protein 27 (HSP27). We then detected HSP27 in sera of patients by use of ELISA, indicating a sensitivity of 100% and a specificity of 84% for the recognition of pancreatic cancer.**

Conclusions: **The detection of DJ-1 and HSP27 in pure defined tissue and the retrieval of HSP27 in serum by antibody-based methods identifies a potential marker for pancreatic cancer.**

Pancreatic cancer is a formidable challenge in oncology and has the lowest 5-year survival rate (~2%) of any solid cancer *(1)*. Only 10% of patients present with a potentially curable tumor. To gain a chance of combating this cancer type, we must elucidate early tumorigenic processes. In current practice, cancer antigen 19-9 assays and imaging techniques are not optimal for detecting small pancreatic lesions. Improved understanding of DNA/RNA alterations and protein concentrations, in combination with the development of high-throughput, sensitive techniques, could lead to the discovery of a panel of biomarkers that will enable aggressive therapy while tumors are still curable *(2)*.

Surface-enhanced laser desorption/ionization (SELDI)[5] is a proteomic high-throughput technique that uses chromatographic surfaces to retain proteins depending on their physicochemical properties, followed by direct analysis via time-of-flight mass spectrometry (MS) *(3)*. This technique requires only a small amount of sample, making it ideal for small biopsies or microdissected tissue (required to produce the homogeneous tissue samples typically used in cancer

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[5] Nonstandard abbreviations: SELDI, surface-enhanced laser desorption/ionization; MS, mass spectrometry; 2-DE, 2-dimensional gel electrophoresis; HSP, heat shock protein; TBS, Tris-buffered saline; IHC, immunohistochemistry.

research). Microdissected tissue material, free of contaminating and unwanted tissue components, is extremely important for producing clean data for biomarker identification in cancer diagnostics and in elucidating clonal heterogeneity of tumors. We were able to show in a previous study that the detection of differentially expressed proteins was possible only in pure microdissected samples *(4)*. In the case of pancreatic cancer, the tumor cells have to be separated from all surrounding tissue constituents. This separation can be done only with an extremely precise technique such as laser-based microdissection. Laser-based microdissection has been combined with ProteinChip technology to identify protein markers in other cancers *(5–11)*.

In this study, we used ProteinChip technology to analyze pure microdissected populations of cells from healthy exocrine pancreatic tissue and the central and peripheral areas of pancreatic adenocarcinomas to detect discriminating specific protein profiles.

Materials and Methods

LASER MICRODISSECTION

We obtained 9 pancreatic central tumor areas (pT2/pT3), matched healthy pancreatic samples (n = 10), and 9 pancreatic tumor margins [mean age 61.1 (SD 6.2) years] after surgical resection with informed consent at the Department of General and Visceral Surgery of the Friedrich Schiller University Jena, Germany. The samples were collected fresh, snap-frozen in liquid nitrogen, and stored at −80 °C. We categorized tumor specimens according to their WHO classification.

Laser microdissection was performed with a laser microdissection and pressure catapulting microscope (Palm) as described elsewhere *(12)*. Briefly, we microdissected ~3000 to 5000 cells each on native air-dried, unstained cryostat tissue sections in a maximum of 20 to 30 min. We extracted proteins by incubating with 10 μL lysis buffer (100 mmol/L Na-phosphate, pH 7.5, 5 mmol/L EDTA, 2 mmol/L $MgCl_2$, 3 mmol/L 2-β-mercaptoethanol, 1 mL CHAPS, 500 μmol/L leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride) for 30 min on ice. After centrifugation (15 min; 13 000*g*), the supernatant was immediately analyzed or frozen in liquid nitrogen for a maximum of 1 day.

PROFILING OF TISSUES

We analyzed the protein lysates from microdissected tissues (central tumor, tumor margin, and healthy tissue) on both strong anion exchange arrays (Q10) and weak cation exchange arrays (CM10; Ciphergen Biosystems) as described *(12)*. We preincubated array spots in a washing/loading buffer containing 100 mmol/L Tris-buffer, pH 8.5, with 0.02% Triton X-100 (for Q10 arrays) and 100 mmol/L Tris-buffer, pH 4.5, with 0.02% Triton X-100 (for CM10 arrays) followed by application of 2 μL sample extract on ProteinChip arrays, which were incubated at room temperature for 90 min in a humidity chamber. After washing 3 times with the same buffer and 2 final washing steps with water, we applied 2 × 0.5 μL sinapinic acid (saturated solution in 0.5% trifluoroacetic acid/50% acetonitrile). We performed mass analysis by use of a ProteinChip Reader (series 4000; Ciphergen) according to an automated data collection protocol. Spectra were normalized with total ion current and cluster analysis of the detected signals. We calculated respective *P* values for healthy pancreatic tissue and pancreatic carcinoma tissue with CiphergenExpress (version 3.0). We selected normalized spectra with signals between 2.5 and 20 kDa for low range and 20 and 200 kDa for high range, exhibiting a signal-to-noise ratio of at least 10, and analyzed them with the Mann–Whitney *U*-test for nonparametric data sets.

TWO-DIMENSIONAL GEL ELECTROPHORESIS

We prepared samples for 2-dimensional gel electrophoresis (2-DE) directly from surgical material of pancreatic tumor and corresponding healthy pancreatic tissue assessed by a pathologist. Proteins were isolated and 2-DE performed as described *(12)*. In brief, isoelectric focusing was carried out on a Multiphor II (Amersham) using 7-cm immobilized pH gradient strips in a pI interval of 3 to 10. Vertical sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed in a Novox MiniGel system (Invitrogen) using 4% to 12% Bis-Tris Zoom™ gel (Invitrogen). The gels were stained with Simply Blue Safe Stain (Enhanced Coomassie; Invitrogen).

IN-GEL DIGESTION

We compared protein patterns of the 2-DE gels from healthy pancreatic and tumor tissue and excised consistently differentially expressed proteins as well as ~95 additional spots. In-gel digestion of proteins was performed as described *(12)*. In brief, excised gel pieces were destained and dried. After rehydration and digestion with 10 μL of a trypsin solution (0.02 g/L; Promega) at 37 °C overnight, we applied supernatants directly on a NP20 ProteinChip array (Ciphergen). An empty gel piece underwent the same treatment as a control. After addition of the matrix (CHCA; Ciphergen), we analyzed peptide fragment masses by use of the ProteinChip reader. The spectra for the peptide-mapping experiments were externally calibrated using 5 proteins including Arg8-vasopressin (1082.2 Da), somatostatin (1637.9 Da), dynorphin (2147.5 Da), ACTH (2933.5 Da), and insulin β-chain (3495.94 Da). We identified proteins using the fragment masses generated through trypsin digestion by searching in a publicly available database (ProFound; http://prowl.rockefeller.edu/prowl-cgi/profound.exe).

IMMUNODEPLETION ASSAY

We incubated 2 μL antihuman heat shock protein 27 (HSP27) polyclonal antibody (SP5105P; Acris) or antihuman DJ-1 monoclonal antibody (ab11251; Abcam) with 10 μL protein A-agarose (Sigma-Aldrich) for 15 min on ice. Pellets were generated by centrifugation, and the supernatants were discarded. The pellets were washed

twice with a buffer containing 20 mmol/L HEPES, pH 7.8, 25 mmol/L KCl, 5 mmol/L $MgCl_2$, 0.1 mmol/L EDTA, and 0.5 mL NP-40. We then incubated 5 μL of a lysate from laser-dissected pancreatic tumor with each pellet for 45 min on ice. We incubated 5 μL of the lysate with protein A-agarose without the specific antibody, as a negative control, for 45 min on ice. After incubation, samples were cleared by centrifugation, and 2 μL of each supernatant was analyzed by use of ProteinChip arrays.

IMMUNOHISTOCHEMISTRY

We placed 8-μm cryostat sections of pancreatic cancer tissue and adjacent healthy tissue on slides, air-dried them for ~60 min at 20 °C, and fixed them in paraformaldehyde as described *(11)*. After fixation, slides were treated in the microwave at 80 watts (3 × 3 min) in 10 mmol/L citric acid, pH 6.0, to inhibit endogenous peroxidase activity. We rinsed them twice with Tris-buffered saline (TBS), pH 7.4, and incubated them overnight at 4 °C in a humidity chamber with a corresponding primary polyclonal antibody against HSP27 (SP5105P; Acris) or a primary antihuman DJ-1 monoclonal antibody (ab11251; Abcam). We rinsed the slides 3 × 10 min in TBS and used the Vectastain Elite ABC reagent set (Vector Laboratories) and the Jenchrom pxbl reagent set (MoBiTec) according to the manufacturer's instructions to visualize antibody localization. Negative controls were incubated with the labeled secondary antibody only. Sections cut in parallel to the immunohistochemistry (IHC)-treated sections were stained by hematoxylin and eosin for better identification of different tissue areas. IHC staining was evaluated by a pathologist.

QUANTIFICATION OF HSP27 BY ELISA

In addition to the tissue samples, we independently tested a set of serum samples from 35 patients (pancreatic tumor; pT2/pT3), taken before surgery at the Department of General and Visceral Surgery of the Friedrich Schiller University Jena and at the Department of General Surgery, University of Heidelberg. The samples were immediately divided into aliquots and frozen at −80 °C. Serum samples from healthy donors (n = 37) were obtained with the same protocol, divided into aliquots, and frozen at −80 °C. The mean (SD) age for the tumor patients was 61.3 (8.1) years, and for the control volunteer group, 45.6 (15.4) years. The set did not include any sera from patients whose samples were used for ProteinChip array analysis.

We measured serum HSP27 concentrations by use of an appropriate ELISA (Sigma-Aldrich) in duplicate, according to the manufacturer's instructions. We measured ELISA plates on a microtiter plate reader (MRX II; Dynex Technology) at 450 nm and calculated concentration of the respective protein in serum according to a calibration curve. We calculated *P* values by 1-sided *t*-test, constructed ROC curves for HSP27 serum concentration by plotting sensitivity vs 1-specificity, and calculated the areas under the ROC curves.

WESTERN BLOT

Identification of HSP27 and DJ-1 was verified by Western blot. We subjected 30 μg crude extract of pancreatic tumor tissue to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrophoretically transferred it to polyvinylidene difluoride membrane (Bio-Rad). Membranes were incubated overnight at 4 °C with a 1:1000 dilution of anti-HSP27 antibody (SP5105P; Acris) or a 1:1000 dilution of anti–DJ-1 antibody (ab11251; Abcam; diluted in 20 g/L milk powder in TBS containing 05 mL/L Tween 20) and for 3 h with the corresponding secondary antibody. Both HSP27 and DJ-1 were detected by alkaline phosphatase reaction. We estimated band intensities for both proteins by visual inspection.

Results

PROTEIN PROFILING OF CENTRAL PANCREATIC TUMOR, TUMOR MARGIN, AND ADJACENT HEALTHY TISSUE

We excised tissue areas corresponding to ~3000 to 5000 cells per probe by use of laser microdissection and pressure catapulting microscope. In this way, we successfully collected 28 samples in total (10 healthy pancreatic samples, 9 central pancreatic tumors, and 9 tumor margins). All protein lysates from the microdissected tissues were applied to both the Q10 arrays and the CM10 arrays and analyzed on a ProteinChip Reader Series 4000. The SELDI measurements of all tissue samples detected up to 340 peaks in the 2.5- to 200-kDa interval, with normalized intensities. After evaluation with CiphergenExpress, a number of these peaks were found to be significantly different between pancreatic carcinomas and healthy pancreatic tissue samples (Table 1).

IDENTIFICATION OF SIGNALS

To separate protein lysates, we subjected histologically checked pancreatic tumor pieces and biopsies derived

Table 1. Significantly different signals that separate pancreatic carcinoma and adjacent healthy pancreatic tissue detected on Q10 arrays (anion exchanger) and CM10 arrays (cation exchanger).

Signal up-regulated in tissue	Molecular mass, Da	Array surface
Healthy	7.961	CM10
Carcinoma	11.171	Q10
Healthy	15.147	CM10
Healthy	15.898	CM10
Carcinoma	19.939[a]	Q10
Carcinoma	22.538	CM10
Carcinoma	22.749[a]	CM10
Healthy	25.083	Q10
Healthy	25.916	Q10
Carcinoma	66.731	CM10
Carcinoma	66.738	Q10
Carcinoma	134.259	Q10

[a] Signals representing DJ-1 and HSP27.

from healthy pancreatic tissue to 2-DE (see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue4). Numerous protein spots showing differential expression were observed. Approximately 95 protein spots in the interval of ~10 to 150 kDa were excised from the gels, and we analyzed peptide fingerprints of the tryptic-digested spots by use of SELDI time-of-flight MS. In this way, we were able to identify 29 proteins by database searching (ProFound; http://prowl.rockefeller.edu/prowl-cgi/profound.exe; see Table 1 in the online Data Supplement).

One of these identified proteins, DJ-1 (see Fig. 1, spot 2, in the online Data Supplement), matched well in molecular mass with a significantly differentially expressed signal detected in prior protein profiling using SELDI. This signal of ~20 kDa was detected on Q10 arrays and showed an increased expression in samples derived from pancreatic tumor. Presence of the spot discriminated significantly between central pancreatic carcinoma and tumor margin and healthy adjacent pancreatic tissue ($P = 3.66 \times 10^{-2}$) as well as between pancreatic carcinoma and healthy pancreatic tissue (2.08×10^{-2}). Another significantly different signal possessing an *m/z* of nearly 23 kDa matched well to a protein identified as HSP27 (see Fig. 1, spot 13, in the online Data Supplement). This significantly different signal was up-regulated in pancreatic carcinoma tissue compared with healthy pancreatic tissue (2.68×10^{-2}) as detected on CM10 arrays in prior protein profiling.

We double-checked that DJ-1 and HSP27 match the differentially expressed peaks at 19.9 and 22.7 kDa by use of ProteinChip analysis with immunodeplete assays, using microdissected pancreas carcinoma tissue as starting material. Analyses showed that the peaks corresponding to DJ-1 and HSP27 were reduced. In the negative control without the specific antibody, these peaks were clearly detectable (Fig. 1).

CHARACTERIZATION BY IMMUNOHISTOCHEMISTRY AND WESTERN BLOT

To further characterize the identified markers and to localize DJ-1 and HSP27 in tissue sections, we examined their expression in several pancreatic tissue samples by immunohistochemistry using specific antibodies against DJ-1 and HSP27. Negative controls without the primary antibody or without any antibody had no signal. Both healthy pancreatic cells and malignant tumor cells demonstrated cytoplasmic signals for DJ-1 and HSP27 in all tissue samples examined, but in every case with higher signal intensities in tumor cells (Fig. 2). Quantitative differences between the expression of these interesting proteins in healthy pancreatic cells and malignant tumor cells were as clear as in ProteinChip array results. Furthermore, we carried out IHC assays to a number of additional proteins, including PEBP, cystatin B, and cyclophilin A (see Fig. 2 in the online Data Supplement).

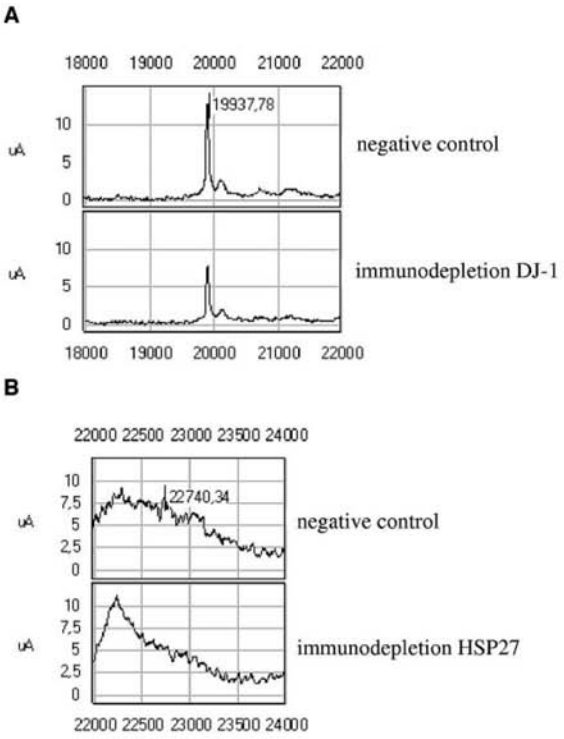

Fig. 1. Immunodepletion assay of microdissected pancreatic tumor tissue.

(*A*), *peak* (19937.79 Da) representing DJ-1 was detectable in the negative control and clearly decreased in the depleted control. (*B*), *peak* (22740.34 Da) representing HSP27 was detectable in the negative control but not in the corresponding depleted probe.

To further confirm that the localized DJ-1 and HSP27 are identical to the peaks found by ProteinChip analysis, areas of similar size from tumorous and healthy tissue that were previously analyzed in IHC were obtained by tissue laser microdissection. In protein lysates from the pancreatic tumor fraction, we detected signals identical in mass to the interesting peaks obtained with the initial SELDI-MS analysis on proper arrays (Q10 and CM10). In the protein lysate from IHC-negative areas, these peaks were absent (Fig. 3). We also examined by Western blot the expression levels of DJ-1 and HSP27 in lysates derived from an independent set of pancreatic tissue specimens (see Fig. 3 in the online Data Supplement). We detected strong signals corresponding to DJ-1 and HSP27 in the majority of analyzed probes.

ANALYSES OF HSP27 IN SERUM

In addition to the tissue samples, we quantified HSP27 in a sample set of sera derived from pancreatic cancer patients and controls (n = 72) by use of an independent ELISA analysis. The concentration of HSP27 in serum from tumor patients was found to be significantly higher

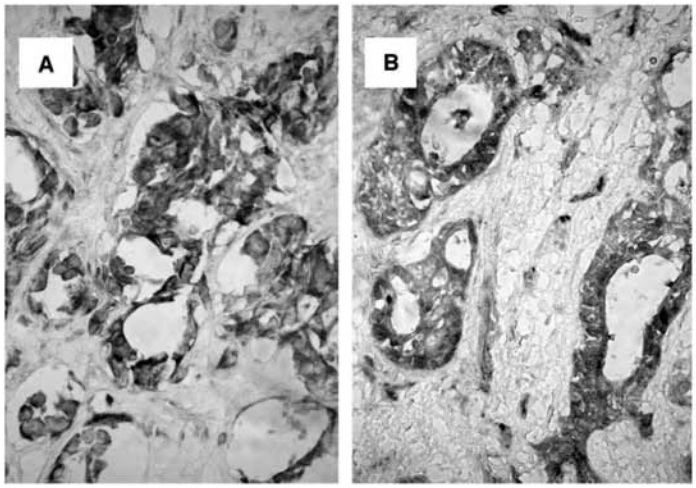

Fig. 2. IHC of DJ-1 (*A*) and HSP27 (*B*) visualized by microscopy (magnification ×400).

Tissue derived from pancreatic carcinoma with increased signal intensity in carcinoma structures.

than in serum from controls ($P < 0.001$). The median for controls was 0.76 μg/L, and for patients with pancreatic carcinoma, 2.93 μg/L (Fig. 4). We constructed ROC curves for HSP27 serum concentration, resulting in a sensitivity of 100% at a specificity of 84% and a cutoff of 1.33 μg/L. The area under the ROC curve was calculated as 0.985 (Fig. 4).

Discussion

Despite enormous efforts, relevant markers useful for screening have been established in only a few tumor types *(13, 14)*, and no studies have found markers for early detection of pancreatic cancer *(15–19)*. 2-DE, especially in combination with microdissection, seems an appropriate tool *(19)*, but proteins in the peptide interval, as well as those of high hydrophobicity or of extreme isoelectric points, are difficult to separate and hence are typically neglected, resulting in a loss of potentially interesting proteins. Additionally, the sensitivity is low compared with MS.

In this study, we used protein-profiling SELDI MS and 2-DE to find biomarkers that might enable earlier tumor detection. Only a small number of protein-profiling studies in pancreas tumor have so far been performed using SELDI technology *(20–22)*. Our study improves on this approach by using samples of pure microdissected cells derived from central pancreatic tumor areas, tumor margin, and adjacent healthy tissue. We detected a small number of signals that discriminated well between the 3 sample groups. For identification of these signals, we separated histologically checked tissue specimens from pancreatic tumors and healthy pancreatic tissue using 2-DE followed by analysis of peptide mass fingerprints using SELDI MS. We excised 95 protein spots from those that were obviously different in 2-DE gels and, using the methodology described in Melle et al. *(23)*, identified 29 proteins unequivocally. Two proteins identified in this

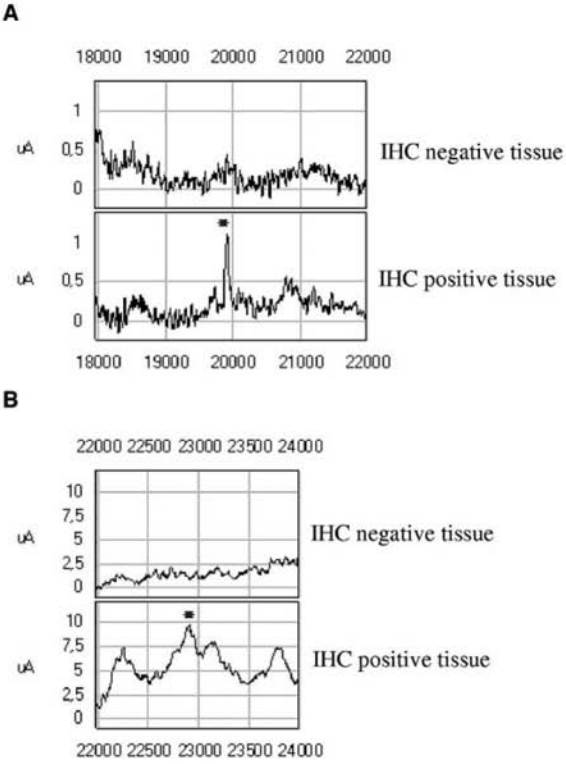

Fig. 3. Reanalysis of IHC-treated tissue sections by ProteinChip technology.

Areas of similar size of healthy and tumorous tissue that were applied in IHC were microdissected and analyzed on proper ProteinChip arrays. (*A*), signal (*) with a molecular mass of ~20 kDa representing DJ-1 was detectable in protein lysate derived from pancreatic tumor tissue on a Q10 array. This signal is absent in the protein lysate from the healthy tissue fraction. (*B*), signal (*) corresponding to HSP27 was detectable in IHC-positive tissue derived from pancreatic tumor tissue on a CM10 array in contrast to the IHC-negative tissue from healthy pancreas samples, where the signal was absent.

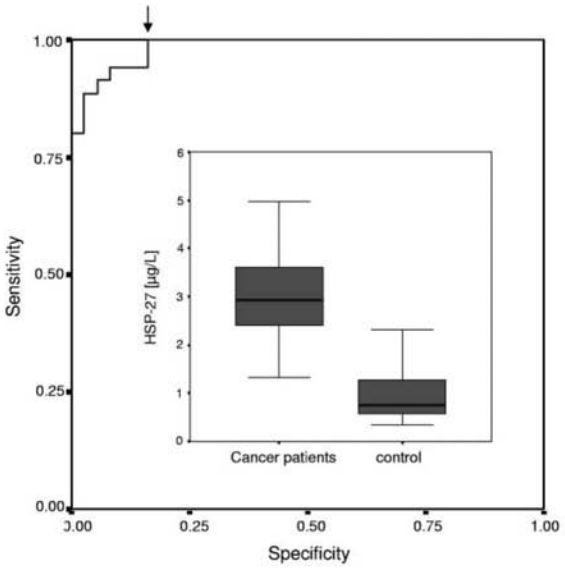

Fig. 4. ROC curve for HSP27 serum concentration for patients with pancreatic carcinoma and controls.

The area under the curve was 0.985 [95% confidence interval (CI) 0.965–1]. At a cutoff of 1.33 µg/L (arrow), the sensitivity was 100% (CI 90.11% to 100%) and specificity 84% (CI 68.86% to 92.35%). The median concentrations for patients and controls were 2.93 and 0.76 µg/L, respectively. *Inset*, *box plot* of serum concentrations of HSP27 for controls and pancreatic carcinoma patients for an independent sample set. The *lines* inside the *boxes* denote the medians. The *boxes* represent the interval between the 25th and 75th percentiles, and the *whiskers* indicate the interval between maximum and minimum.

manner, DJ-1 and HSP27, matched well to different signals found in prior protein-profiling assays. Both proteins were up-regulated in pancreatic cancer and discriminated well between central tumor and tumor margin vs healthy tissue and between central tumor and healthy tissue. We confirmed the identities of both proteins in immunodepletion assays and further characterized them by immunological techniques. In an independent set of pancreatic tumor tissue specimens that had not been assessed earlier by ProteinChip technology, we also detected strong signals corresponding to DJ-1 and HSP27 by Western blot analysis. We also analyzed the identified HSP27 specifically in serum by a corresponding ELISA, with exactly the same results for HSP27 as found in prior protein profiling.

Our evidence suggests that the concentration of DJ-1 is increased in pancreatic carcinomas and that this increase distinguishes pancreatic tumor tissue from adjacent healthy tissue. DJ-1 is a conserved protein, coded by the gene *PARK7* (Parkinson disease 7),[6] that is reported to be involved in diverse cellular processes including cellular transformation, control of protein–RNA interaction, oxidative stress response, and control of male infertility. The *PARK7* gene is associated with an autosomal recessive, early onset Parkinson disease *(24)*. Recent reports show that *PARK7* is overexpressed in a number of cancer types, including breast, lung, and prostate. It partly obtains its transforming activity by an RNA helicase named Abstrakt *(25, 26)*. In primary breast cancer samples, DJ-1 negatively regulates the *PTEN* tumor suppressor and thus produces overexpressed hyperphosphorylation of PKB/Akt and increased cell survival *(27)*. In a proteomic analysis of gastric cancer, DJ-1 was detectable only in metastatic tumor tissue vs nonmetastatic tumor tissue and healthy gastric tissue *(28)*. Based on this fact, it seems likely that DJ-1 contributes to the metastatic potential of a tumor.

HSP27 is a powerful molecular chaperone whose main function is to prevent the aggregation of nascent and stress-accumulated misfolded proteins. It is able to interact directly with various components of the tightly regulated programmed cell death machinery, upstream and downstream of the mitochondrial events, and seems to play a role in the proteasome-mediated degradation of selected proteins. HSP27 is associated with poor prognosis in gastric, liver, and prostate carcinoma and osteosarcomas *(29, 30)*. Data concerning the prognosis potential of HSP27 in the above cancer types are conflicting because a recent study showed that in gastric cancer, HSP27 was not detectable in metastatic tumors and could be found only in samples derived from nonmetastatic tumors *(28)*. To date, only a few studies are available that report an association of differential expression of HSP27 and pancreatic carcinoma, and the results of these investigations are partly conflicting *(31, 32)*.

Whereas our study identified DJ-1 and HSP27 as potential new biomarkers for the early detection of pancreatic cancer, further studies with larger sample sizes using cancerous and healthy tissue acquired by noninvasive sampling methods are required.

In conclusion, we show that a proteomic procedure composing tissue microdissection, protein profiling by ProteinChip technology, separation and identification of interesting proteins by 2-DE, peptide mass fingerprinting, and SELDI MS as well as confirmation of these proteins using immunological techniques is able to identify and characterize differentially expressed proteins that could serum markers for pancreatic carcinoma. The clinical relevance of these findings will require further study.

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[6] Human gene: *PARK7*, Parkinson disease 7.

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2.4 Kapitel 4: Comparative proteomic analysis of normal and tumor stromal cells by tissue on chip based mass spectrometry (toc-MS)

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Diagnostic Pathology 2010, 5:10.

Die Konzentration der vom Tumor abgegebenen Proteine verringert sich, je weiter man vom Ursprungsort entfernt nach ihnen sucht. Deshalb ist eine Suche am Ursprungsort, dem Tumor, unabdingbar. Um Biomarker besser funktionellen Bereichen im Gewebe zuordnen zu können, entwickelten wir eine Technik, mit der es erstmals möglich war, 100-500 stromale Zellen, die aus Hals-Kopf-Tumor nahen, bzw. Normalgewebe nahen Regionen stammten, massenspektrometrisch zu analysieren. Aufgrund der geringen Zellzahlen innerhalb des Stromas konnten hier potentielle Stromamarker für Hals-Kopf-Tumore detektiert, aber noch nicht identifiziert werden. Ob es sich hierbei um Marker für Metastasierung handelt, wird in weiteren Versuchen geklärt.

Die Probenvorbereitung, Mikrodissektion und massenspektrometrische Vermessung wurde in Zusammenarbeit mit Günther Ernst und Annett Bleul durchgeführt. Die massenspektrometrische und biostatistische Auswertung erfolgten von mir persönlich. Alle anderen Autoren beteiligten sich beratend im Rahmen eines Verbundprojektes an der Arbeit.

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DIAGNOSTIC PATHOLOGY

SHORT REPORT Open Access

Comparative proteomic analysis of normal and tumor stromal cells by tissue on chip based mass spectrometry (toc-MS)

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Abstract

In carcinoma tissues, genetic and metabolic changes not only occur at the tumor cell level, but also in the surrounding stroma. This carcinoma-reactive stromal tissue is heterogeneous and consists e.g. of non-epithelial cells such as fibroblasts or fibrocytes, inflammatory cells and vasculature-related cells, which promote carcinoma growth and progression of carcinomas. Nevertheless, there is just little knowledge about the proteomic changes from normal connective tissue to tumor stroma. In the present study, we acquired and analysed specific protein patterns of small stromal sections surrounding head and neck cell complexes in comparison to normal subepithelial connective tissue. To gain defined stromal areas we used laser-based tissue microdissection. Because these stromal areas are limited in size we established the highly sensitive 'tissue on chip based mass spectrometry' (toc-MS). Therefore, the dissected areas were directly transferred to chromatographic arrays and the proteomic profiles were subsequently analysed with mass spectrometry. At least 100 cells were needed for an adequate spectrum. The locating of differentially expressed proteins enables a precise separation of normal and tumor stroma. The newly described toc-MS technology allows an initial insight into proteomic differences between small numbers of exactly defined cells from normal and tumor stroma.

Findings

Carcinoma tissue does not only consist of tumor cells but also of fibroblasts, endothelial cells or vascular structures, and inflammatory cells forming the so-called desmoplastic stroma reaction or supportive tumor stroma. Many steps in carcinoma development e.g. proliferation, angiogenesis, invasion and metastasis are promoted by microenvironmental factors produced by these stromal cells. It is well known that the reciprocal interactions between tumor and stroma cells, i.e., cancer associated fibroblasts (CAF), tumor endothelial cells (TEC) and tumor associated macrophages (TAM) result in tumor progression. The close vicinity of CAFs to the cancer cells enhance tumor growth by secreting growth factors like transforming growth factor beta (TGF beta), matrix degrading enzymes like matrix metalloproteinases (MMP) and angiogenic factors such as vascular endothelial growth factors (VEGF) [1]. The investigation of those microenvironmental factors at the proteomic level requires a technical workflow that enables the isolation of small defined areas of stroma on the one hand and a sufficient high sensitivity to analyse these small amounts of cells on the other hand. One part of this attempt is the laser-based tissue microdissection [2]. Hereby, small areas of interest can be easily separated from the remaining tissue and further analyzed with genomic or proteomic approaches. The second prerequisite for the proteomic analysis of stromal cells is a highly sensitive detection technique. Gel-based techniques do not meet this requirement but mass spectrometry by MALDI (matrix assisted laser desorption and ionization) seems to be a better choice as shown in several studies using microdissected tissue [2,3]. Using affinity chromatographic surfaces SELDI (surface enhanced laser desorption and ionization) offers the highest sensitivity - but with low resolution - and is a commonly used tool to investigate differentially expressed proteins

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in body fluids, cells and tissue [4-9]. In general, SELDI is useful to compare crude protein lysates with a high sensitivity; MALDI, in contrast, displays a higher resolution which is useful for the identification of proteins. So far, after microdissection about 3000-5000 cells are needed to receive an adequate proteomic profile. Nevertheless, it is tedious to reach even this cell number from small stromal areas within a tumor. Therefore, the purpose of this study was to develop and refine a proteomic technique which is sensitive enough to analyse as few as a hundred microdissected cells.

Microdissection of stroma from normal and tumor tissue

All head and neck tumor samples (n = 14) and normal controls (n = 14) were obtained after surgical resection at the ENT (Ear, Nose, Throat) Department of the University Hospital Jena; they had been collected fresh, snap frozen in liquid nitrogen, and were stored at -80°C. Tumor specimen were categorized to the WHO classification criteria [10]. Ethical approval was obtained from the local Research Ethic Committee.

From these samples 12 μm cryostat sections were prepared. One section was stained with hematoxylin-eosin (HE) and examined microscopically in order to detect tissue areas of interest for microdissection (see [11]). A corresponding unstained tissue section was mounted on a microscope slide coated with a 1.35 μm membrane (polyethylene naphtalate (PEN) Zeiss/Palm, Bernried, Germany). Tissue areas from normal and tumor stroma (approx. size 300 × 300 μm) containing approximately 100 to 500 cells were cut out and moved by a laser microdissection and pressure catapulting microscope (LMPC; Zeiss/Palm, Bernried, Germany) or a fine needle directly on ProteinChip arrays (Fig. 1). For catapulting, a microplasma is induced under the dissected tissue area. This plasma lifts the piece of tissue to a reaction cup or to a ChipArray fixed by a special mount, each. For regular formed tissue pieces with more than 100 cells we found that it is more secure to attach the dissected area to a fine needle and deposit it elsewhere under microscopically control.

Applying microdissected tissue onto ProteinChip arrays and mass spectrometric analysis

A Q10 ProteinChip array (strong anion exchanger; BioRad) was activated (see [11]) and wetted with 0.5 μl lysis buffer (100 mM Na-phosphate (pH 7.5), 5 mM EDTA, 2 mM $MgCl_2$, 3 mM 2-β-mercaptoethanol, 0.1% CHAPS, 500 μM leupeptine, and 0.1 mM PMSF). Under a stereo microscope (Stemi 2000c, Zeiss) the tissue section was placed on the spot of the ProteinChip array. Tissue lysis on spot was performed for 1.5 h at 4°C in a humidity chamber. After lysis and incubation the spots were washed three times with 5 μl of a washing/binding buffer (100 mM Tris-buffer, pH 8.5 with 0.02% Triton X-100) and rinsed 2 times with water. 2 × 0.5 μl sinapinic acid (saturated solution in 0.5% TFA/50% acetonitrile) was applied as matrix on the dried spots. The matrix which co-crystallizes with proteins absorbs the laser energy and transfers part of its charge to the proteins. Mass analysis was performed in a ProteinChip Reader (PCS 4000, Ciphergen Biosystems Inc, Fremont, CA) with a manual data collection protocol.

Because cells were microdissected, placed and lysed directly on the spot of the ProteinChip array under control of a stereo microscope, we named this technique 'tissue on chip based mass spectrometry' (toc-MS). Areas of different size and cell number were tested. At least 100 cells were needed for an adequate spectrum. For the analysis of the normal and tumor samples 300 cells were dissected for more robust results. Compared to the SELDI standard procedure the sensitivity is increased at least tenfold and, because no protein lysis and extraction is needed, time of analysis is shorter by half. In contrast to MALDI imaging, which allows to analyse spatial resolved protein spectra over tissue sections and other mass spectrometry techniques, the SELDI characteristic affinity chromatograhic chip surfaces allow a more quantitative analysis of proteins.

Bioinformatic analysis of mass spectrometry data

The resulting protein profiles between 2 kDa to 20 kDa (low range) and 20 kDa to 200 kDa (high range) were subjected to CiphergenExpress™ Client 3.0 software (CE) and a cluster and rule-based data mining algorithm (XLminer 3.0, BioControl Jena GmbH). The CE software was used for the processing of raw spectra and the calculation of *P*-values and cluster plots. In the low range we found 8 peaks with a *P*-value lower 0.05. In the high range 5 peaks with this characteristic could be found. The two most significant proteins for the low and high range are displayed in box plots in Figure 2.

The 7,477 Da peak is significantly higher expressed ($P = 0.0003$) in tumor stroma, while the 80,044 Da peak ($P = 0.0009$) is reduced in tumor stroma. An initial data base search according molecular size offered for the 7,477 Da mass the beta defensin 119 (UniProtKB/Swiss-Prot Q8N690 Chain: 22-84: 7493 Da). Human beta-defensines (HBD) are cationic, antimicrobial peptides produced by epithelial cells and show altered inconsistent expression in cancers [12,13]. Analyses of their expression in tumor stroma are not published yet. For the fibroblast growth factor 23 only a role in phosphate homeostasis and related disorders is known [14]. The protein with a molecular mass of 80,044 Da is equivalent in size to the unphosphorylated ski oncogene (UniProtKB/Swiss-Prot P12755, 80,005 Da) which was discovered as oncogene by its ability to transform chicken embryo fibroblasts upon overexpression. But in newer studies also anti-oncogenic activities are discussed (for review see [15]).

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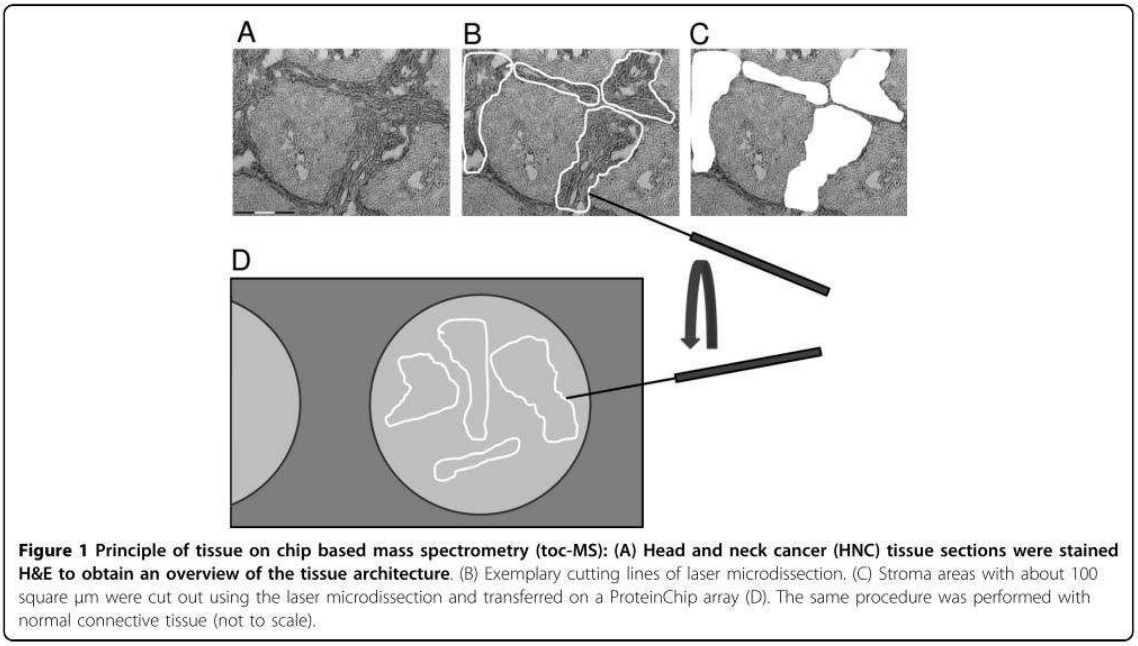

Figure 1 Principle of tissue on chip based mass spectrometry (toc-MS): (A) Head and neck cancer (HNC) tissue sections were stained H&E to obtain an overview of the tissue architecture. (B) Exemplary cutting lines of laser microdissection. (C) Stroma areas with about 100 square µm were cut out using the laser microdissection and transferred on a ProteinChip array (D). The same procedure was performed with normal connective tissue (not to scale).

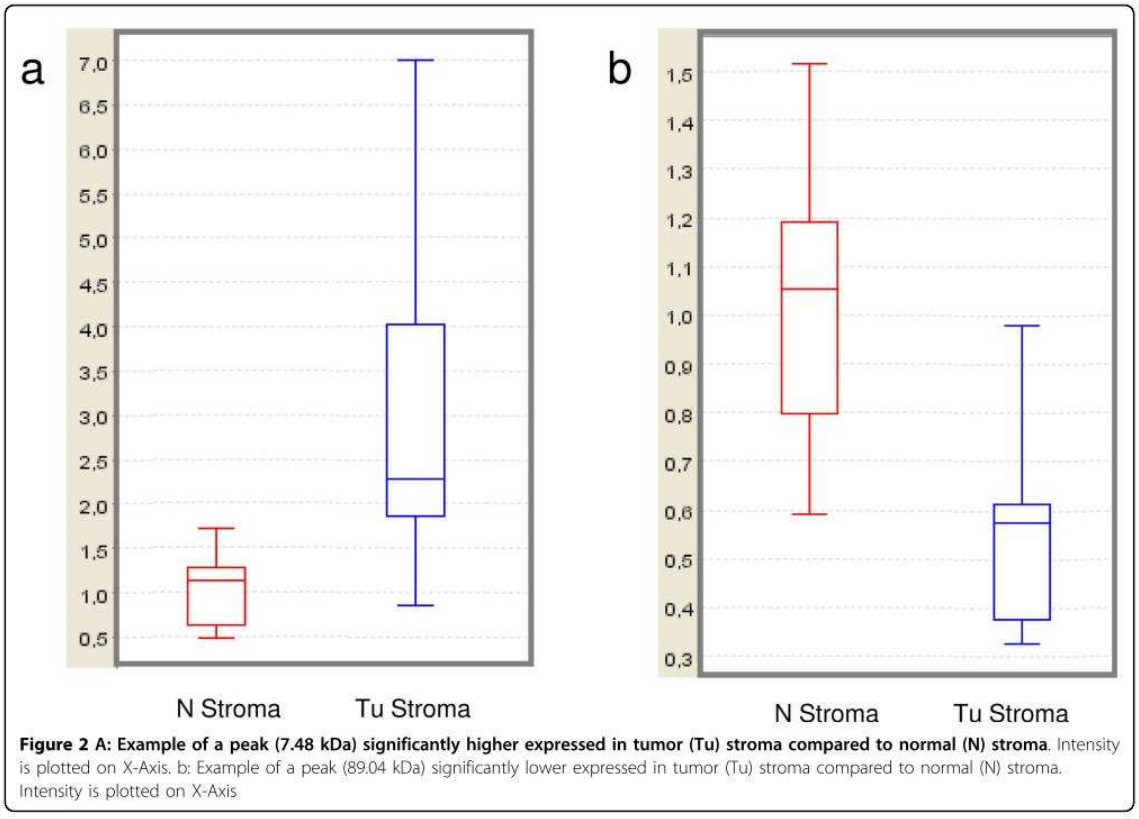

Figure 2 A: Example of a peak (7.48 kDa) significantly higher expressed in tumor (Tu) stroma compared to normal (N) stroma. Intensity is plotted on X-Axis. b: Example of a peak (89.04 kDa) significantly lower expressed in tumor (Tu) stroma compared to normal (N) stroma. Intensity is plotted on X-Axis

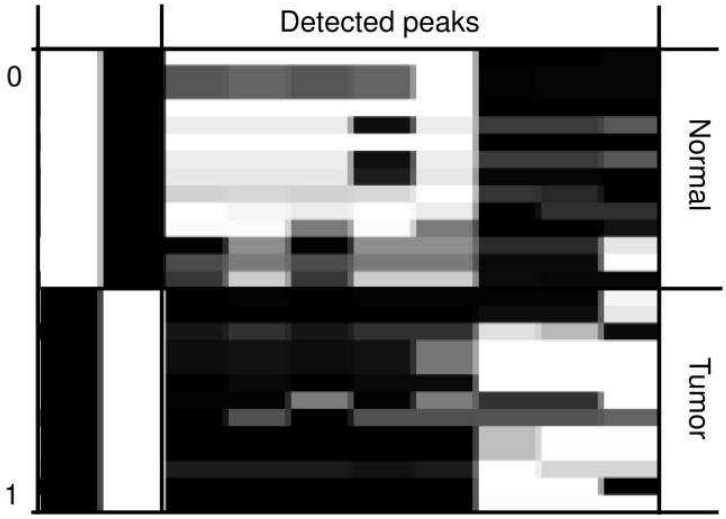

Figure 3 XLminer heat map including stroma from normal (n = 14) und tumor (n = 13) samples (horizontal rows). In columns the relevant features (peaks) are displayed. The analysis resulted in a sensitivity of up to 92.8% and a specificity of 100%.

The subsequent modified fuzzy c-means data analysis algorithm underlying the XLminer software [5] consists of three steps in particular allowing adequate analysis of small sample groups. The clustering step, the rule extraction and rating step, and the rule-base construction step finally result in a heat-map and in values for sensitivity and specificity separating both groups. The analysis of all tumor and normal samples with XLminer resulted in a sensitivity of up to 92.8% and a specificity of 100% (Fig. 3).

In conclusion, we applied toc-MS successfully to analyse a few hundred stromal cells quantitatively and to differentiate between those stromal areas near to tumor and to normal epithelium. An exact identification of these proteins with tryptic digestion and tandem MS is in progress. Ongoing research focuses on down-scaling the procedure to a higher sensitivity.

Abbreviations

MS: mass spectrometry; toc-MS: tissue on chip based mass spectrometry; MALDI: matrix-assisted laser desorption and ionization; SELDI: surface enhanced desorption and ionization; CAF: cancer associated fibroblasts; TEC: tumor endothelial cells; TAM: tumor associated macrophages; MMP: matrix metalloproteinases; LMPC: laser microdissection and pressure catapulting.

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Authors' contributions

NE performed SELDI experiments. GE performed tissue microdissection. CM supervised SELDI experiments and did database research. AB performed pathological examination and classification of tissues; critical reading of manuscript. JHC performed conception, single cell work, data collection and writing manuscript. KJ performed conception, interpretation of data and writing manuscript. KF performed conception, biochemical work, interpretation of data and writing manuscript. OGL looked for adequate tumor samples and clinical aspects and writing manuscript. FvE performed conception, design, supervision and writing manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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2.5 Kapitel 5: “Confirmation of the biological significance of transthyretin as a biomarker for CTCL by its protein interaction partners

Niko Escher, Robert Kob, Martin Kaatz, Christian Melle, Christina Hipler, Ferdinand von Eggeling
In Vorbereitung zur Einreichung bei Proteome Science

Proteine üben ihre Funktion selten allein aus, sondern bilden oft große Netzwerke oder Signalkaskaden. In diesem Paper wird die These aufgestellt, das es möglich sein sollte, Biomarker mittels Proteininteraktionsstudien zu charakterisieren und anhand dieser Interaktionspartner neue Biomarker zu finden. Durch eine Modifikation der *blue native* Polyacrylamidgelelektrophorese (BN-PAGE) war es erstmals möglich, gezielt Protein-Protein-Interaktionen im Serum nachzuweisen. Dem differentiell exprimierten Protein Transthyretin (TTR), welches in Patienten mit Mycosis Fungoides (MF) stark herunterreguliert ist, konnten Interaktionspartner wie Apolipoprotein A1, Apolipoprotein A4, Retinol bindendes Protein 4 (RBP-4), und der Retinoid X Rezeptors (RXR-β) zugeordnet werden. Hierbei konnte Apolipoprotein A1 ebenfalls in MF herunterreguliert nachgewiesen und validiert werden.

Die Etablierung der BN-PAGE erfolgte in Zusammenarbeit mit Robert Kob. Alle anderen praktischen Arbeiten wurden von mir persönlich durchgeführt. Die anderen Autoren wirkten beratend oder durch Bereitstellung von Probenmaterial an dieser Arbeit mit.

Confirmation of the biological significance of transthyretin as a biomarker for CTCL by its protein interaction partners

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Abstract

Previously, we described transthyretin (TTR) and its posttranslational modifications as a downregulated marker in mycosis fungoides (MF), a benign subtype of cutaneous T-cell lymphoma (CTCL). In order to understand the biological role of this protein in the etiology of this disease more precisely, it is essential to identify further interacting proteins, which clarify the pathways of progression.

In this study, we combined for the first time, blue native polyacrylamide gel-electrophoresis (BN-PAGE) with surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) to detect on the one hand new TTR interaction partners and on the other to prove whether these TTR interaction partners can be used as biomarkers themselves.

Thus we identified apolipoprotein A1, which was additionally found downregulated in the serum of MF patients, apolipoprotein A4, retinol binding protein 4 (RBP-4), and retinoid X receptor β (RXR-β) as interaction partners of TTR. The RXR family plays a role in cell differentiation and proliferation and is known to be the target of bexarotene, which is used in the treatment of CTCL.

The combination of BN-PAGE and SELDI-TOF-MS allowed the detection of protein interaction partners, which in the case of RBP-4 and RXR builds a connection between the common tumour marker TTR and tumour progression in CTCL.

Introduction

Mycosis fungoides (MF) is a subtype of cutaneous T-cell lymphoma (CTCL) and represents at a proportion of about 50% the most common disease in this group, followed by CD30+ lymphoproliferative disorders and Sézary syndrome [1]. MF typically starts in middle adulthood and is clinically divided into patch, plaque, and tumour stages [2]. About 25% of MF patients with extensive patches or plaques will develop progressive disease. To date, only a few biomarker candidates have been identified for this disease. Next to neopterin, β2-microglobulin, and soluble IL-2 receptor have been described as possible candidate markers that are elevated in CTCL patients [3,4].

In our most recently reported study, we detected and identified transthyretin (TTR) and its modifications as downregulated biomarkers in the serum of MF patients [5]. So far, TTR is known to be the major carrier for serum thyroxine (T4) and triiodothyronine, and it can also be found in cerebrospinal fluids [6]. The transport of retinol (vitamin A) via its interaction with retinol binding proteins (RBPs) is also facilitated by TTR. Nevertheless, it is important to identify further biomarkers that may be useful for the diagnosis of CTCL patients.

Until now, biomarkers are mostly detected or identified by an undirected search in a patient's serum, blood cells, or tissue [7–10]. Another hitherto unused way to find new biomarkers is the identification of protein interacting partners of an already known biomarker. This is of particular significance, because nearly all proteins have multiple interaction partners, and the up- or downregulation of a protein in a complex protein network should also have consequences for its interacting proteins.

Currently, protein–protein interactions can be studied by techniques like yeast two-hybrid screening, affinity chromatography, and immunoprecipitation [11,12]. Using these techniques, the purification of protein complexes is either adapted to capturing the target using antibodies or indirect immobilization through tagged proteins. For immunoprecipitation, it is necessary to have an antibody with high affinity and sensitivity. Furthermore, the epitopes can be hidden in a protein complex or the antibodies can block the binding of an interaction partner, which might play an essential role in the biological function of this complex. Therefore, it is necessary to use a technique that avoids these limiting factors.

Schagger et al. [13] originally described blue native polyacrylamide gelelectrophoresis (BN-PAGE). This technique allows the separation of protein complexes, first in a native dimension followed by the separation of interacting proteins in a second denaturing

dimension. This technique has already been used for the analysis of murine intestinal brush border membranes, chloroplasts, and membrane proteome analysis of the green sulfur bacterium Chlorobium tepidum [14–16]. Interaction partners found with BN-PAGE can be ideally proven and identified with surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS). This technique combines on-chip processing of biological samples with mass spectrometry and is most commonly used for the detection of biomarkers in body fluids or tissues [9,17]. However, a few studies identifying new protein–protein interactions using this technique have also been reported [12,18,19].

Combining both BN-PAGE and SELDI for the first time, we found apolipoprotein A1, apolipoprotein A4, retinol binding protein 4 (RBP-4), and fragments of retinoid X receptor β (RXR-β) as TTR interaction partners. Comparing these TTR interaction partners with a list of differentially expressed proteins from previous studies (unpublished data), we have identified apolipoprotein A1 as a downregulated biomarker in the serum of MF patients.

Materials and Methods

BN-PAGE

For the first dimension, serum was diluted 1:2 with native PAGE sample buffer (50 mM bis-Tris, 50 mM NaCl, 10% (w/v) glycerol, 0.001% Coomassie Brilliant Blue G-250 Serva, pH 7.2). BN-PAGE was performed using a gradient gel (4.5%–15%) with a specific running buffer (50 mM bis-Tris, 50 mM tricine, pH 6.8) plus 3 ml cathode buffer (running buffer containing 0.4% Serva Blue G). Electrophoresis was conducted at 130 V until the ion front migrated to the gel bottom (Fig. 1A).

For the second dimension, specific complexes from two equivalent lanes were excised from the stained gradient gel. These pieces were transferred into reaction tubes and covered with equilibration buffer (1% SDS, 1% β2-mercaptoethanol). Subsequently, the gel bands were incubated at 95 °C in SDS Laemmli buffer for five minutes and transferred into the wells of two SDS gels (B). Electrophoresis was conducted at 180 V, and subsequently one gel was transferred for western blot analysis of TTR to determine which lane contained the separated TTR complex. Finally, the corresponding lane of the second-dimension gel was stained with Coomassie Brilliant Blue G-250 (SERVA). The resulting bands were excised from the gel and digested with trypsin. The resulting peptides were finally analyzed using gold ProteinChip arrays and SELDI-TOF-MS (C).

Tryptic digestion

The protein bands were transferred into reaction tubes and incubated in 50% methanol with 10% acetic acid for 2 h. Subsequently the gel pieces were transferred through a graded series of buffers finally resulting in dehydration of the gel pieces. The dehydrated pieces were subsequently incubated with 20 ng/ml trypsin overnight at 37 °C, and the resulting digest was applied to N20 ProteinChips and analyzed using SELDI-TOF-MS. Finally, the resulting spectra were interpreted using a search of the ProFound database (http://prowl.rockefeller.edu).

Coimmunoprecipitation of the TTR–apolipoprotein A1 complex

For coimmunoprecipitation (CoIP), protein A-agarose was incubated with 5 µl anti-TTR antibody (Sigma, anti-human prealbumin, goat) for 45 minutes. Subsequently the beads were washed and 50 µl of human serum was added. After 45 min incubation, bound proteins were eluted using 15 µl SDS buffer (40 mM Tris-HCl, pH 6.75 containing 4% SDS, 10% β2-mercaptoethanol, 40% glycerin, and 0.002% bromphenol blue) at 95 °C for 5 min. The eluted proteins were loaded onto a 12% SDS polyacrylamide gel and separated by electrophoresis under 160 V running conditions and subsequently blotted using 150 mA for 2 h. The protein-blot membrane was then washed, nonspecific binding sites were blocked using 3% milk powder and the treated membrane was incubated with anti-apolipoprotein antibody (Abcam, goat, polyclonal) overnight. A secondary antibody (anti-goat), conjugated to alkaline phosphatase, was incubated with the western blot membrane for 3 h.

Immunodepletion

About 10 µl of protein A-agarose was washed with CoIP buffer: 20 mM HEPES, containing 0.1 mM EDTA and 50 mM KCl. Anti-apolipoprotein A1 antibody (3 µl; Abcam, goat, polyclonal) was coupled to the protein A by incubation with the agarose conjugate at 4 °C for 45 min. After blocking with 3% milk powder, the protein A-agarose was washed with CoIP buffer, and 4 µl of 1:50 diluted serum was added. The supernatant was removed and applied to a copper sulfate activated IMAC 30 ProteinChip array. A negative control using a nonspecific IgG antibody was treated similarly.

Results

Discovery of TTR protein interaction partners

In previous studies, we demonstrated that TTR and its modifications are differentially expressed in the serum of patients with MF compared with a healthy control group [5]. To assess the function of TTR and to detect new biomarkers, we performed TTR interaction partner studies using a combination of BN-PAGE and SELDI-TOF-MS. To do this, serum was loaded onto a native gradient gel (first dimension) at different concentrations. Nine bands from two equally loaded lanes were excised and transferred to nine wells of two SDS polyacrylamide gels (second dimension). The first gel was blotted onto a membrane, and a specific band representing TTR was detected in one lane. Subsequently this section of the second SDS-gel was stained with Coomassie Brilliant Blue G-250. All bands were excised and transferred to individual vials for tryptic digestion. The resulting peptide mass fingerprints analyzed by SELDI-TOF-MS were used in a search of the ProFound database (http://prowl.rockefeller.edu/). We could identify TTR and four TTR interaction partners, namely apolipoprotein A1, apolipoprotein A4, RBP-4, and RXR-β. Finally, we checked a list of differentially expressed proteins from previous studies after ProteinChip profiling and found a protein with a molecular mass of 28.1 kDa downregulated in the serum of MF patients (Fig. 2).

Coimmunoprecipitation

To confirm the BN-PAGE finding that TTR and apolipoprotein A1 are complexed in human serum, a CoIP study was conducted. To do this, specific anti-TTR antibody, or as a negative control IgG, was coupled to protein A-agarose. After incubation with serum, bound proteins were eluted and subjected to SDS-gel separation before western blotting using a specific anti-apolipoprotein A1 antibody for immunodetection. While there was a band visible in the precipitate using the specific antibody for the light chain of the primary antibody, this band is missing in the control using nonspecific antibodies (Fig. 3). Thus we confirmed a protein–protein interaction between TTR and apolipoprotein A1.

Immunodepletion of the differentially expressed proteins

To confirm apolipoprotein A1 as a biomarker in the serum of patients with MF, an immunodepletion study was conducted. To do this, anti-apolipoprotein A1 antibody was conjugated to protein A-agarose. The conjugated antibody was incubated with serum for 45 min at 4 °C, and the supernatant was applied to a copper sulfate activated IMAC 30 ProteinChip array. A negative control using nonspecific IgG was similarly treated. The protein profiles were analyzed by SELDI-MS. In the experiment using the specific apolipoprotein A1 antibody, the peak with a molecular mass of 28.1 kDa was depleted compared with the negative control using nonspecific IgG (Fig. 4).

Discussion

Although treatable in the early stages, most types of cancer become more aggressive as they progress. For CTCL, biomarkers are needed to diagnose this disease in the early stages so that suggestions regarding its therapy may be given. About 25% of MF patients with extensive patches or plaques will develop progressive disease. To understand the mechanism of progression, it is not only important to identify new biomarkers but also necessary to obtain information regarding the function of these biomarkers, because understanding the proteomic network is a prerequisite for finding new therapies for this disease. Therefore, this work aimed at the identification of TTR interaction partners on the one hand and the analysis of these interaction partners on the other, to determine whether they can serve as biomarkers themselves.

In a previous study, we detected the differential expression of TTR and its modifications in the serum of MF patients and a healthy control group [5] and demonstrated the ability of SELDI-TOF-MS to detect differentially expressed proteins. Furthermore, we showed that SELDI allows not only the detection of proteins but also their modifications as distinct from other techniques used such as 2D-PAGE or ELISA.

In the present study, we combined for the first time the SELDI-TOF technique with BN-PAGE to detect TTR interaction partners and to analyze whether the newly detected interacting proteins can be used as diagnostic biomarkers. Thus we identified four TTR interaction partners, namely apolipoprotein A1, apolipoprotein A4, RBP-4, and RXR-β. We further compared the molecular masses of the interacting proteins with the molecular masses we had found earlier for proteins that are expressed differentially between MF patient serum and control serum from an unaffected group of people. Apolipoprotein A1 corresponds well to the downregulated protein (molecular mass 28.1 kDa) as detected by SELDI analysis. An immunodepletion study confirmed that apolipoprotein A1 corresponds to the 28.1 kDa downregulated protein. In the present study, we describe for the first time the protein interaction of TTR and apolipoprotein A1 in a complex derived from MF serum and the identification of downregulated apolipoprotein A1 in MF compared with healthy control serum. While TTR can be found down- or upregulated [5,6,21], apolipoprotein A1 is found downregulated in other diseases [7,22].

TTR is the major carrier for T4 and triiodothyronine that can also be present in cerebrospinal fluids [6]. The transport of retinol via its interaction with RBPs is also facilitated by TTR. An interaction between TTR and apolipoprotein A1 has been also been described by Sousa et al. [20] in the high-density lipoprotein fraction of plasma.

Both proteins are expressed in the liver, and hitherto there has been no correlation between the downregulation of these proteins in CTCL or any other diseases.

The most surprising finding was the presence of RXR-β in the serum. Retinoids like retinoic acid receptors (RAR) and RXRs inhibit proliferation and induce differentiation in melanoma cells [23]. Both receptor types exert their biological function through three subtypes (α, β, and γ). Nuclear retinoid receptors are ligand-dependent transcription factors that bind to cis-acting DNA sequences, known as retinoid acide response elements and retinoid X response elements, which are present in the promoter regions of retinoid responsive target genes. Abnormalities in the expression and function of both RAR and RXR play an important role in the growth of various cancers [24]. Retinoids comprise a group of structural and functional analogs of vitamin A (retinol). RBP is the specific carrier of retinol in blood and transports it from the liver to the target tissues. An interaction between RBP and TTR in hepatocytes before their secretion into the bloodstream has already been mentioned by Bellovino et al. [25]. We have shown an interaction between TTR and RBP-4. Moreover, Soares et al. [26] reported RBP-4 to have influence in the Portuguese TTR V30M amyloid polyneuropathy.

Finally, the RXR can also be regulated via a pharmacological response to bexarotene. The RXR specific retinoid bexarotene is used in the treatment of CTCL [27]. Therefore, changes in the serum level of TTR and of its interaction partners, which are responsible for retinoid binding to its receptors RAR or RXR, might have consequences for cell proliferation.

The combination of BN-PAGE and SELDI-TOF-MS allows the detection of endogenously expressed protein interaction partners, which in the case of RBP-4 and RXR build a connection between the common tumor marker TTR and tumor progression in CTCL.

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Figures

Fig. 1: Combining BN-PAGE and SELDI-TOF-MS. In the BN-PAGE first dimension (A), serum was loaded at different concentrations on a native gel. The gel was stained with Coomassie Brilliant Blue G-250, and stained and separated protein complexes from two equally loaded lanes were transferred to two denaturing second-dimension SDS gels that separate the protein complexes into single proteins. One of these gels was western blotted with TTR immunodetection to determine which lane contained the TTR complex, and the other was stained with Coomassie Brilliant Blue G-250 (B). Subsequently, the stained bands from the lane containing the TTR complex were excised for tryptic digestion. Finally, the digest was analyzed using SELDI-TOF-MS.

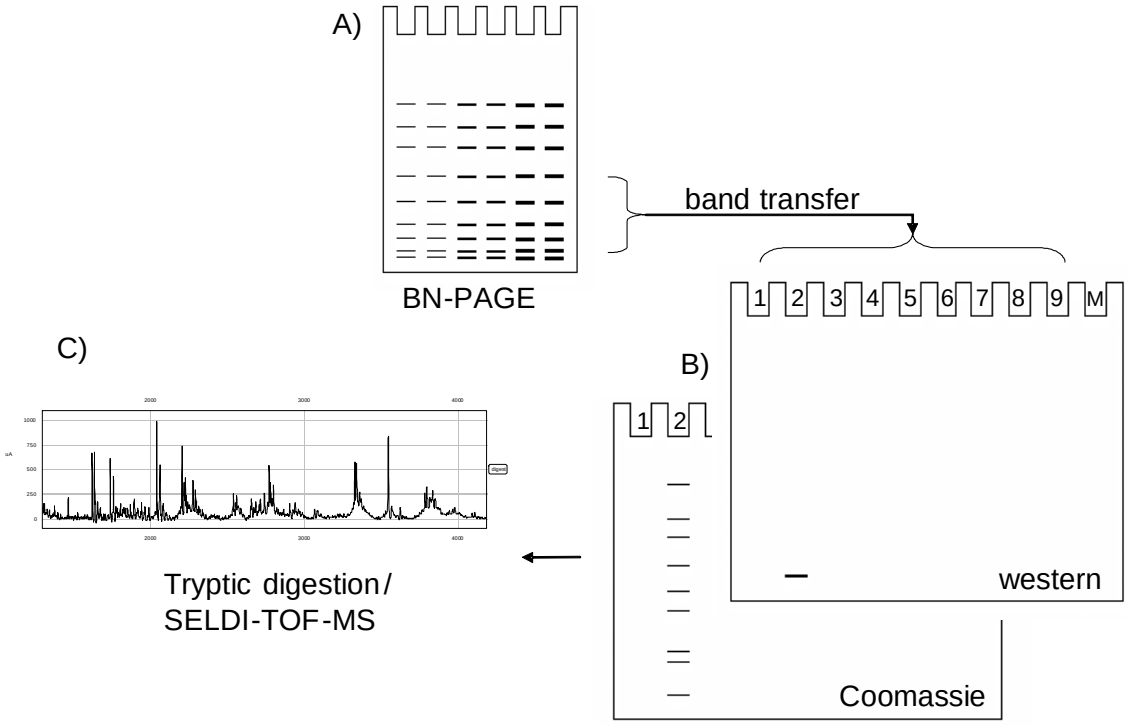


Fig. 2: Cluster plotting revealed a p value of 2.87 × 10–6 when comparing serum from MF patients with healthy control serum for the 28.1 kDa protein peak.

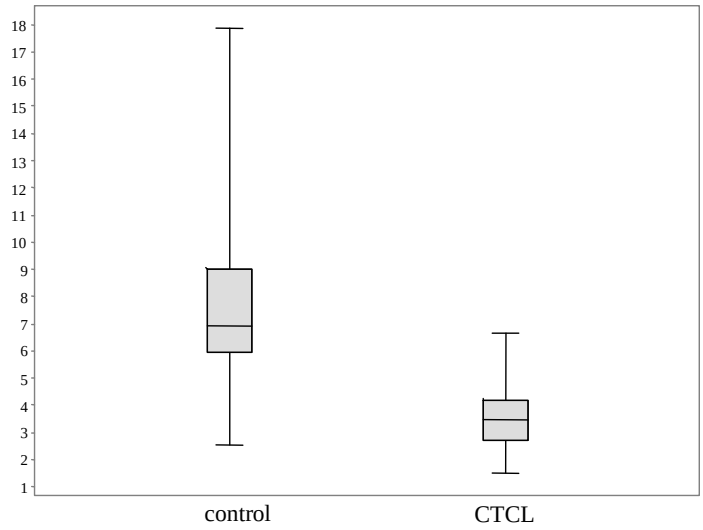


Fig. 3: Coimmunoprecipitation of apolipoprotein A1. Specific anti-TTR antibody and nonspecific IgG were coupled to Protein A-agarose beads and incubated with serum. After washing with Co-IP buffer, bound proteins were transferred for SDS-PAGE. Co-IP using the TTR antibody shows a specific band, while the control Co-IP using nonspecific IgG lacks the signal.

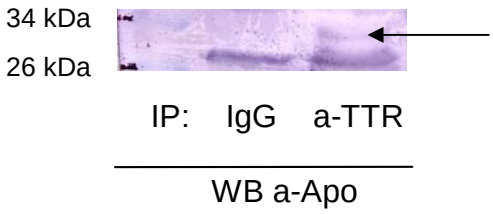
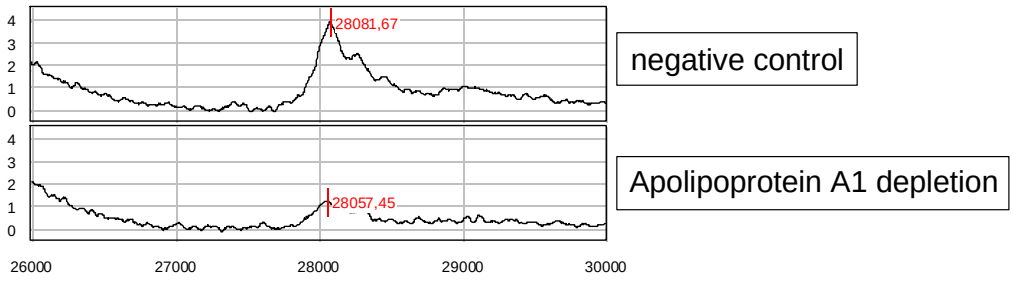


Fig. 4: Immunodepletion of apolipoprotein A1. For immunodepletion, a specific anti-apolipoprotein A1 antibody was coupled to Protein A-agarose. The beads were incubated with serum, and the depleted sample was applied to a copper-activated IMAC 30 ProteinChip array. While the peak with a molecular mass of 28.1 kDa is present in the negative control using a nonspecific antibody, the signal from the sample incubated with beads bound to specific anti-apolipoprotein antibody is depleted in this region.



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2.6 Kapitel 6: Various Members of the E2F Transcription Factor Family Interact in vivo with the Corepressor Alien

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Im Kampf gegen Tumore oder andere Krankheiten ist es nicht nur wichtig, Biomarker zu identifizieren, die eine klare und möglichst auch frühe Diagnose und Behandlung ermöglichen. Eine molekulare Charakterisierung oder neue Ansatzpunkte für mögliche Medikamente sind ebenso von enormem Nutzen. Bei der Proliferation von normalen aber auch Tumorzellen spielt der Zellzyklus eine entscheidende Rolle. Dieses Paper zeigt, das Mitglieder der E2F-Transkriptionsfaktor-Famile nicht nur mit dem Protein Alien interagieren, sondern dieses auch reprimierend auf E2F1 wirkt und somit ein neues und wichtiges Mitglied in der Regulation des Zellzyklus darstellt.

Die Identifizierung der Interaktionen zwischen Mitgliedern der E2F Familie und Alien wurde von mir durchgeführt und in Zusammenarbeit mit Robert Kob validiert. Funktionelle Analysen wurden in Zusammenarbeit mit Stephan Tenbaum und Michael Eisold durchgeführt.

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Various Members of the E2F Transcription Factor Family Interact *in vivo* with the Corepressor Alien

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Proteins perform their activities in cells by the cooperation within protein complexes. For this reason, it is important to investigate protein−protein interactions to receive insights in physiological processes. A multitude of proteins are involved in the regulation of the cell cycle. Specific key factors participating here are members of the E2F transcription factors. Using an *in vivo* protein−protein complex detection assay, which comprises mass spectrometric and immunological techniques, we detected a number of known as well as new protein−protein interactions. We describe here for the first time protein complexes containing the corepressor Alien and members of the E2F transcription factor family. Furthermore, we assessed the functional relevance and show a repression of the transcriptional activity of E2F by Alien. Additionally, we detected new interactions that link endogenously expressed Alien with the tumor suppressor retinoblastoma protein (pRB) and with proteins involved in cell cycle regulation.

Keywords: E2F transcription factor • co-repressor Alien • cell cycle regulation • protein−protein interactions • SELDI-MS • immunoprecipitation

Introduction

Proteins rarely work alone; they are commonly organized in protein complexes by protein−protein interactions to accomplish their functions in cells. Interacting proteins are often involved in the same cellular processes, and thus, the identification of interacting partners of a given protein with unknown function may give insight into the physiological role of this protein.[1] A biological pathway that concentrates a multitude of proteins in several high specific protein complexes is the eukaryotic cell cycle.[2−4]

Members of the E2F transcription factor family are particularly involved in the regulation of the cell cycle progression and crucial for the G1/S transition and DNA replication.[5,6] Therefore, E2Fs interact in temporal and spatial dependent manner with several specific proteins.[7−9] Because E2Fs are involved in several cellular processes, it is conceivable that a multitude of interacting partners are yet unknown. Thus, it is necessary to detect protein interacting complexes containing endogenous E2Fs *in vivo*. Hereby, it is important to apply approaches that minimize the detection of false positive protein interactions. For this purpose, we used a recently described *in vivo* protein−protein complex detection assay.[10] This assay combines a mass spectrometric technique, the surface-enhanced laser desorption ionization (SELDI), and immunological methods for detection and identification of endogenous protein complexes. Using this protein−protein complex detection assay, we were able to assess a number of known as well as yet unknown protein interactions. Interestingly, a complex containing E2F-1 and the co-repressor Alien was detected. This interaction was confirmed by co-immunoprecipitation experiments and *in vitro* binding assays. Alien was previously characterized as a co-repressor for specific members of the nuclear hormone receptor superfamily including the thyroid hormone and vitamin D3 receptor.[11−13] Alien interacts in a hormone-sensitive manner with these nuclear receptors and mediates transcriptional repression. Thus, we further investigated a potential functional relevance of this interaction and revealed repression of the transcriptional activity of E2F by Alien. Furthermore, among those we identified for the first time novel *in vivo* protein−protein interactions for Alien with endogenously expressed proteins such as pRB and factors involved in cell cycle regulation.

Experimental Procedures

Cell Culture. The human osteosarcoma cell line U-2OS and the cervix carcinoma C33A cell line were cultured in DMEM supplemented with 10% fetal bovine serum.

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For protein−protein interaction experiments, U-2OS cells were grown to 80% confluence and were passaged at a split ratio of 1:4. Cells were harvested at 70−90% confluence and lysed in a buffer containing 100 mM sodium phosphate pH 7.5, 5 mM EDTA, 2 mM $MgCl_2$, 0.1% CHAPS, 500 μM leupeptin, and 0.1 mM PMSF. After centrifugation (15 min; 15000 rpm), the supernatant was immediately used.

Protein−Protein Complex Detection Assay. The protein−protein complex detection assay was described elsewhere.[10] Briefly, 20 μL of Interaction Discovery Mapping (IDM) beads (Ciphergen Biosystems Ltd., Fremont, Ca) were incubated with 4 μL of protein A (Sigma) overnight at 4 °C. A pellet was generated by centrifugation, and the supernatant was discarded. The pellet was washed twice with a buffer containing 50 mM sodium acetate pH 5.0. Afterward, the beads were incubated in a buffer containing 0.5 M Tris/HCl pH 9.0, 0.1% Triton X-100 for 2 h at room temperature for blocking residual reactive groups. The beads were washed three times with 1× PBS. Thereafter, 8 μg of specific antibody against human E2F-1 (H-137, rabbit polyclonal; Santa Cruz Biotechnology), or human E2F-3 (N-20, rabbit polyclonal; Santa Cruz Biotechnology), respectively, or normal rabbit IgG (Pepro Tech Inc.; Rocky Hill, NJ) as negative control, in 50 mM sodium acetate pH 5.0 were applied to the beads and allowed to bind at room temperature for 1 h in an end-over-end mixer. Unbound antibody was removed by washing in 50 mM sodium acetate. Afterward, the beads were washed in 1× PBS, 0.1% Triton X-100, and in 1× PBS and incubated with 100 μL of crude U-2OS cell extract over night at 4 °C in an end-over-end mixer. The unbound proteins were washed away by sequential washes in PBS, 0.5 M sodium chloride, 0.05% Triton X-100 in PBS, PBS, and aqua bidest. Bound proteins were eluated from the IDM beads by 25 μL 50% acetonitrile/0.5% trifluoroacetic acid and gently vortexed for 30 min. Five microliters of the eluated samples were applied to the activated, hydrophobic surface of an H50 ProteinChip Array (Ciphergen Biosystem Inc.; Fremont) and dried on air. After washing with 3 μL aqua bidest, 0.5 μL sinapinic acid (saturated solution in 0.5% TFA/50% Acetonitrile) was applied twice and the array was analyzed in a ProteinChip Reader (series 4000; Ciphergen, Fremont) according to an automated data collection protocol by SELDI-MS. This includes an average of 265 laser shots to each spot with a laser intensity of 2300 and 3500 nJ, respectively, dependent on the measured region (low = 2.5−20 kDa and high = 20−200 kDa) and an automatically adapted detector sensitivity.

Peptide Fingerprint Mapping. Peptide fingerprint mapping was carried out as described elsewhere.[15] In brief, the volume of eluated samples was reduced to a maximum of 10 μL using a speed-vac (ThermoServant) and subjected to SDS-PAGE for separation of containing proteins followed by staining with Simply Blue Safe Stain (Enhanced Coomassie, Invitrogen) or by silver staining (SilverQuest, Invitrogen). Specific gel bands were excised, destained, and dried followed by rehydration and digestion with 10 μL of a trypsin solution (0.02 μg/μL; Promega) at 37 °C over night. The supernatants of the in-gel digestions were applied directly to NP20 arrays (Ciphergen). After addition of the matrix (CHCA), peptide fragment masses were analyzed using the ProteinChip Reader, series 4000 instrument. A standard protein mix (all-in-1 peptide standard mix; Ciphergen), including Arg8-vasopressin (1082.2 Da), somatostatin (1637.9 Da), dynorphin (2147.5 Da), ACTH (2933.5 Da), and insulin beta-chain (3495.94 Da) was used for calibration. Proteins were identified using the fragment masses searching in a publicly available database (http://129.85.19.192/profound_bin/WebProFound.exe).

Coimmunoprecipitation. The CoIP assays were carried out as described.[10] Briefly, a specific antibody or, as negative control, normal rabbit IgG were bound on protein A-agarose beads. Crude extract (250 μL) from U-2OS cells was incubated with the antibody loaded beads for 1 h at 4 °C. Then the resins were washed three times with CoIP buffer containing 20 mM HEPES/KOH pH 8.0, 50 mM KCl, 0.1 mM EDTA and 0.05% CHAPS. Bound proteins were subjected to 10% SDS−PAGE and detected by immunoblotting. (For details concerning used antibodies, see the Figure legends).

Transient Transfection Experiments. C33A cells were cultured and transfected in DMEM supplemented with 10% FCS. Cells were seeded 48 h prior to transfection into 6 well plates at a density of 240 000 cells per well. Medium was changed 9 h prior to transfection. DNA was introduced according to the $CaPO_4$ transfection method described.[16] E2F1-promoter-luc or E2F1mut-promoter-luc reporter-plasmids (0.5 μg),[17] 0.7 μg indicated expression-vector and, 0.3 μg pCMV-LacZ per well were used, and the total amount of DNA was adjusted to 5.4 μg with calf thymus DNA. Twenty-four hours past transfection cells were washed 3 times with 2 mL PBS and medium was changed. After further 48 h of culturing, cells were harvested and lysed to measure luciferase as well as beta-galactosidase activity for normalization and transfection efficiency control. Independent triplicate experiments were performed and repeated at least twice.

GST-Pulldown. GST-pulldown experiments were performed as described before.[16] Briefly, GST or GST-hAlien were bacterially expressed in the strain BL21(lys) via induction of the tac-promoter by adding 0.2 mM isopropyl- b-D-thiogalactopyranoside (IPTG) to the culture medium and incubation for 3 h at 37 °C. Proteins were purified on a glutathione-sepharose resin (Amersham Biosciences) and interaction studies were performed with *in vitro* translated (TNT kit, Promega Corp.) ^{35}S-methionine-labeled pRB or ^{35}S-methionine-labeled E2F-1, respectively. Bound proteins were separated on SDS-gels and stained with Coomassie brilliant blue to ensure equal loading of GST fusion proteins. Bound and labeled proteins were detected by fluorography.

Yeast Two-Hybrid Experiments. The yeast 2 hybrid experiments were performed as described earlier.[11,16] RB cDNA and the RB mutant Rb706 were kindly provided by Dr. D. C. Dean.

Results

Detection of a Physical Interaction Between E2F-1 and the Corepressor Alien. E2F transcription factors are involved in a number of cellular processes. Therefore, E2F proteins interact with a number of specific partners to achieve their several functions. It is conceivable that some of these interacting partners are not yet discovered. For this reason, we first performed a protein−protein complex detection assay to detect interacting proteins of endogenously expressed E2F-1 in crude extracts of U-2OS cells. E2F-1 containing protein complexes were captured by a specific antibody that recognized E2F-1 coupled to IDM beads followed by elution of the captured proteins and analysis of the complex composition using SELDI-MS (Figure 1A). Hereby, among other specific signals a peak possessing a m/z of 49 101 was detected, which roughly corresponds to the MW of E2F-1. In each case, when using a specific anti-E2F-1 antibody, a signal was captured that was only slightly higher than the calculated MW of E2F-1. Until now,

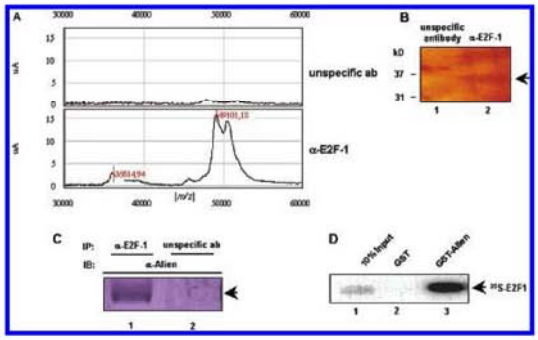

Figure 1. Detection and identification of the corepressor Alien as specific interacting protein of E2F-1 by protein−protein complex detection assay. (A) Anti-E2F-1 antibody was coupled on IDM beads and incubated with U-2OS cell extract. Bound proteins were analyzed by SELDI-MS. Among a signal corresponding E2F-1, a signal at approximately 36 kDa was detectable which is absent in the assay using a unspecific antibody. (B) Eluted proteins from IDM beads were subsequently subjected on a SDS-PAGE for separation and a specific band at approximately 36 kDa (labeled by an arrow) was excised and used for a tryptic in-gel digestion. The peptide mass fingerprints generated by this way were analyzed by SELDI-MS and used for a database quest. Alien was obtained as the best candidate (see also supplemental Figure 1, Supporting Information). (C) For confirmation of the E2F-1−Alien interaction, coimmunoprecipitation experiments were used. Thereby, a specific antibody against E2F-1 was capable to precipitate (IP) Alien from U-2OS cells (labeled by arrow) shown in an immunoblot (IB) (lane 1). Using an unspecific antibody as control, no signal corresponding to Alien was detectable (lane 2). (D) GST-pull-down experiments were used to analyze the *in vitro* binding of E2F-1 with Alien. Bacterial expressed GST or GST-Alien were incubated with reticulate lysate generated *in vitro* translated and ^{35}S-methionine labeled E2F-1. After SDS-PAGE, bound proteins were detected by X-ray films.

we have had no explanation for this fact. Besides the E2F-1 signal, we captured a specific signal of approximately 36 kDa in at least three independent assays. Both signals that derived from E2F-1 and that at 36 kDa were absent in the negative control using an unspecific antibody. For identification of the 36 kDa signal, we subjected the eluted proteins to SDS-PAGEs. In the silver stained gel, we detected a specific band in the range of approximately 36 kDa. Thus, we confirmed the presence of a specific E2F-1−interacting protein. The negative control using an unspecific antibody did not show a band at that position (Figure 1B). This specific band was excised from the gel and was subsequently subjected to an in-gel digestion by trypsin and protein identification. As a control, an empty gel piece underwent the same treatment. The digest yielded solution was spotted on a NP20 array and the peptide mass fingerprints were determined by the ProteinChip Reader. Database searches (Profound; http://129.85.19.192/profound-_bin/WebProFound.exe) revealed the corepressor Alien as the best candidate with an estimated Z-score of 2.43 (supplemental Figure 1). The molecular weight corresponds to the Alien alpha isoform.[18] To confirm this protein complex containing E2F-1 and Alien, coimmunoprecipitation experiments (CoIP) were performed using crude U-2OS cell extract. In line with the previous results, we were able to precipitate Alien using protein A-agarose beads with a specific anti-E2F-1 antibody (Figure 1C). In the negative control using an unspecific antibody coupled on the beads, a signal corresponding to Alien was not detectable. Additionally, we were able to precipitate E2F-1 by a specific anti-Alien antibody in a reciprocal CoIP (supplemental Figure 2, Supporting Information). This suggests that endogenous E2F-1 and endogenous Alien exist in a protein complex. As a further confirmation of this protein−protein interaction, we were able to detect *in vitro* a specific binding of E2F-1 with Alien in GST- pull-down experiments (Figure 1D). Thus, our data suggest that the E2F-1 transcription factor interacts directly with the corepressor Alien.

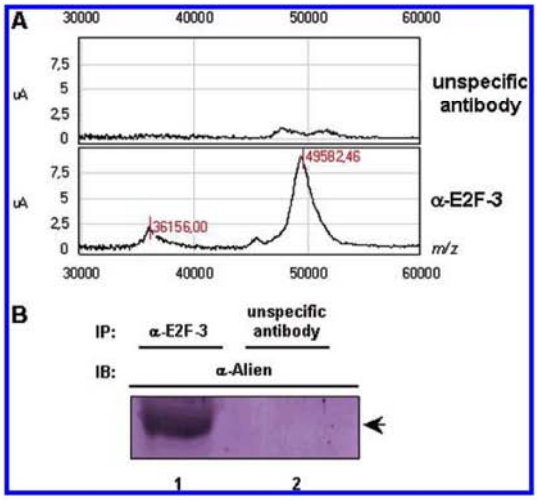

Figure 2. Co-repressor Alien interacts with further members of the E2F transcription factor family. (A) In protein−protein complex detection assays using a specifc anti-E2F-3 antibody, specific signals corresponding to E2F-3 (*m*/*z* 49 582) as well as Alien (*m*/*z* 36 156) were detected in U-2OS cell extract. (B) Protein interaction between E2F-3 and Alien was confirmed in U-2OS cells by a CoIP using an anti-E2F-3 antibody (N-20, rabbit polyclonal; Santa Cruz Biotechnology) for precipitation and an anti-Alien antibody (rabbit polyclonal Pep-Ak2)[14] for immunoblotting. A signal corresponding to Alien is labeled by an arrow (lane 1). As control, CoIP experiments using an unspecific antibody for precipitation this signal is absent (lane 2).

Alien Interacts Physically with Additional Members of the E2F Transcription Factor Family. To investigate whether the corepressor Alien interacts also with other members of the E2F transcription factor family protein−protein complex detection assays using anti-E2F-3 coupled IDM beads were carried out (Figure 2A). Besides the peak at *m*/*z* 49 582 that corresponds well to the MW of E2F-3, we were able to detect again a signal at 36 kDa, which would match to the MW of Alien. To assess a possible interaction between both endogenously expressed E2F-3 and Alien, we performed CoIP-experiments using a specific anti-E2F-3 antibody to immunoprecipitate (IP) protein complexes from crude U-2OS cell extract (Figure 2B). The immunoblot (IB) using an Alien specific antibody resulted in a clear signal in the CoIP with the anti-E2F-3 antibody. In CoIP-experiments using an unspecific antibody as control, no signal that corresponds to Alien was detectable. This suggests that Alien interacts in addition to E2F-1 also with E2F-3. To investigate protein interactions of Alien with other members of the E2F family, additional CoIP experiments using specific antibodies against E2F-2, E2F-3, E2F-4, E2F-5, as well as E2F-6 were employed (supplemental Figure 3, Supporting Information). In these immunoblots, clear signals of Alien were

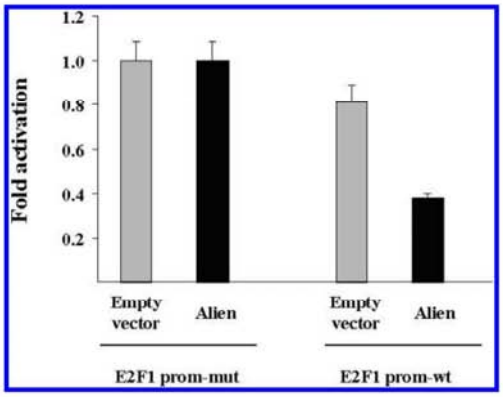

Figure 3. Alien inhibits the E2F-1 promotor activity through E2F binding sites. C33A cells were transfected with the wildtype (E2F1prom-wt) E2F-1 promoter-luciferase reporter (0.5 μg) or with a mutated E2F-site (E2F1prom-mut) together with expression plasmids (0.7 μg) coding Alien or, as a control, the empty vector. The luciferase units were normalized with the β-galactosidase activity derived from the co-transfected pCMV-lacZ reporter plasmid (0.3 μg). The values of the control were set arbitrarily as one. The mean of the three transfection experiments with the deviation is indicated.

detectable compared to the CoIP-control using an unspecific antibody. Thus, taken together, Alien is complexed *in vivo* with various members of the E2F family.

Alien Represses the Transcriptional Activity of E2F-1. To get insight into if the protein interactions between Alien and E2F transcription factors possess a biological function, we assessed whether Alien influences the transcriptional activity of E2F-1. One known important negative regulator of the E2F-1 transcriptional activity is the retinoblastoma protein (pRB).[7] For that purpose, C33A cells were chosen to investigate the impact of Alien on E2F-1 promoter transcriptional activity. These cells lack expression of pRB and thus avoid an overlapping influence of pRB inhibitory activity. C33A cells were transfected with the wildtype E2F-1 promoter-luciferase reporter containing E2F-binding sites or, as a control, with the mutated E2F-binding sites in the same promoter context together with expression plasmids coding for Alien or, as a control, the empty vector. The reporter gene assay showed an inhibition of the transcriptional activity of the E2F-1 promoter activity if the expression plasmid coding for Alien was co-transfected (Figure 3). In contrast, using the reporter with a mutated E2F-site as control, the reporter gene assay resulted in no change of transcriptional activity of the promoter activity. This indicates that Alien represses specifically E2F-mediated transcriptional activity. In an additional experiment, the wild-type E2F-1 promoter-luciferase reporter was co-transfected together with expression plasmids coding for Alien or, as a control, the empty vector and with the E2F-1 expression plasmid (E2F-1) or control vector (Control) (supplemental Figure 4, Supporting Information). Hereby, co-transfected E2F-1 shows a clear activation of the reporter gene. Co-transfection of Alien inhibited also in this case the transcriptional activity. Taken together, Alien represses E2F-mediated transcriptional activity.

Alien Creates Interactions with Proteins Involved in the Cell Cycle Regulation. Next, we focused on detection of additional protein interactions regarding the Alien corepressor protein. Hereby, we were able to identify for the first time new interacting partners of Alien. In the eluted sample derived from a protein−protein complex detection assay using the Alien specific antibody, we confirmed by mass spectrometry the presence of Alien (data not shown). In addition, the immunoprecipitates were separated by SDS-PAGE leading to the detection of a specific protein band at approximately 115 kDa (Figure 4A). This band was treated by protein digestion as mentioned above, and peptide mass fingerprints were generated to be used for database quest (Profound). As the best candidate of this search, we interestingly obtained the retinoblastoma-associated protein p107 with an estimated Z-score of 0.46 (supplemental Figure 5, Supporting Information). p107 was confirmed as an interacting partner of Alien by CoIP experiments (Figure 4A). This indicates Alien is complexed with p107 and is complexed with pRB. To confirm the interaction of endogenous Alien with endogenous pRB, CoIP experiments were employed. In addition to p107, interestingly we detected for the first time protein interactions between Alien and pRB as well as between Alien and CDK2 (Figure 4B and supplemental Figure 3). The interaction of pRB, a member of the pocket protein family, with Alien was further confirmed by the yeast 2-hybrid system (Figure 4C) and in vitro GST-pull-down experiments (data not shown), suggesting a direct interaction between these two proteins. In yeast 2-hybrid experiments, interaction leads to increase in β-galactosidase activity (Miller units). Hereby, an intact pocket domain of pRB was identified to be required for interaction with Alien. Interestingly, the pRB-mutant with an amino acid exchange in the pocket domain of pRB, pRB706, that lacks silencing function[19] also lacks interaction with Alien (Figure 4C). Thus, Alien is also in a complex with pRB and is complexed with factors known to interact with pRB.

Discussion

An opportunity to get insight in cellular processes is to analyze the composition of protein complexes that are involved in these processes. Proteins may exist in several complexes to accomplish distinct functions. Analyses of the interacting partners will provide a strong insight into the physiological role of a particular factor. Therefore, it is essential to identify ideally all interacting partners of proteins *in vivo* to precisely be able to define their biological function. The here-presented data were gained based on a recently described assay for the detection and identification of *in vivo* protein−protein interactions.[10] Investigations of protein−protein interactions using *in vitro* techniques are prone to detect false positives.[20] Also, other *in vivo* methods, for example yeast 2-hybrid assays, can generate a fairly high number of both false positives and negatives.[21] For these reasons, we focused our investigations on protein complexes of endogenously expressed proteins only. The protein−protein complex detection assay used here combines mass spectrometric with immunological approaches. We used a special kind of mass spectrometry named surface-enhanced laser desorption ionization (SELDI).[22] Although SELDI-MS does not have the mass accuracy of other mass spectrometry techniques, it has the advantage to enable the measurement of high m/z ranges and the detection of signals with high molecular masses.[23] In addition, this technique has repeatedly shown to be reproducible.[24,25] There is some evidence that SELDI-MS is also able to perform peptide mass fingerprints from tryptic peptide digests and that despite its lower mass resolution it can provide high probability identification due to a superior peptide coverage.[26,27]

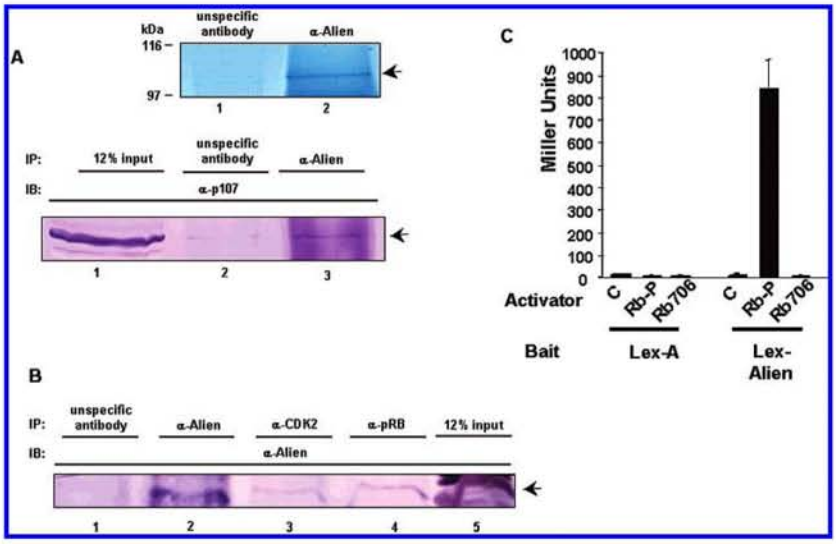

Figure 4. Alien interacts specifically with several proteins that are involved in the cell cycle regulation. (A, top) Eluted proteins from IDM beads coupled with a specific antibody against Alien (rabbit polyclonal Pep-Ak2)[14] or, as control, an unspecific antibody and incubated with U-2OS cell extract were subsequently subjected on a SDS-PAGE for separation and a specific band at approximately 110 kDa (labeled by an arrow) was excised and used for a tryptic in-gel digestion. The peptide mass fingerprints generated by this way were analyzed by SELDI-MS and used for a database quest. As the best candidate p107 was obtained (see also supplemental Figure 5). (A, bottom) In a CoIP using the anti-Alien antibody for protein precipitation from U-2OS cell extract, a specific signal appeared in the immunoblot by an anti-p107 antibody (C-18, rabbit polyclonal; Santa Cruz Biotechnology) (lane 3). This signal is absent in the negative control (lane 2). (B) In different CoIP assays using protein A-agarose beads, coupled antibodies against Alien (rabbit polyclonal Pep-Ak2)[14] (lane 2), CDK2 (D-12, mouse monoclonal; Santa Cruz Biotechnology) (lane 3), or pRB (IF8, mouse monoclonal, Santa Cruz Biotechnology) (lane 4) that were incubated with U-2OS cell extracts a specific signal corresponding to Alien was detectable in the immunoblot which was absent in the CoIP assay using an unspecific antibody (lane 1). (C) Alien α interacts with pRb in the yeast 2-hybrid system. The C706F mutation of the pRb pocket domain abrogates these interactions. Yeast 2-hybrid system[13] was performed with the wildtype retinoblastoma pocket domain (RB−P) or the C706F mutation of RB found in cancer patient lacking silencing function fused to the activation domain B42. As control B42 expression vector was used. As the DNA binding fusion Lex-A alone, as control, or Lex-A Alien was used. Miller units describe the β-galactosidase reporter activity as a measure for interaction.

First, we assessed protein interactions of the E2F-1 transcription factor. E2F-1 is a key factor in the G1/S transition of the cell cycle and controls genes that are involved in DNA replication as well as nucleotide biosynthesis and belonging to the "activating" E2Fs.[5] A multitude of protein interactions containing E2F-1 are available.[28−30] Because of the complexity of the E2F-1 functions, it is conceivable that several E2F-1 complexes exist with some interacting proteins being still unknown. Using the protein−protein complex detection assay, we were capable to identify unequivocally Alien as an interaction partner of E2F-1. Due to the results generated by an *in vitro* GST-pull-down experiment using purified proteins we suggest that E2F-1 and Alien interact directly. Because Alien is described as a corepressor,[11] we investigated the possibility whether Alien has an effect on the transcriptional activity on E2F binding sites. One negative regulator of E2F-1 is the retinoblastoma protein (pRB).[7] For this reason, we used the pRB-negative cell line C33A for a reporter gene assay to avoid possible inhibitory effects triggered by pRB modulating the transcriptional activity of E2F. In the reporter gene assay, we detected a clear inhibition of E2F transcriptional activity by Alien. Thus, taking the data together it suggests that Alien is complexed with E2F-1 and acts as a corepressor for E2F. Because we detected protein interactions between endogenous Alien and other members of the E2F transcription factor family in CoIP experiments, namely the "activating" E2F-2 and E2F-3 as well as the "repressive" E2F-4, E2F-5, and E2F-6, it is tentative to speculate that Alien might exhibit also effects to the activities of the other E2Fs. Most of the above-mentioned E2F proteins have only strong homology in the core domains that mediate DNA binding or dimerization with DP. The dimerization with DP is responsible for the specific recognition of the DNA binding site by E2F.[31] For this reason, it is hypothetically possible that Alien is complexed with the E2Fs within these both domains. Whether the DNA binding function of E2F is disturbed by binding of Alien or Alien represses E2Fs by recruitment HDAC-activity[11] remains to be elucidated. E2F-6 diverges considerably from the other E2F family members as it lacks the domains that are known to mediate transactivation and the pocket protein binding properties of the other mentioned E2Fs and it is unable to perform either of these functions.[32,33] It might be interesting to clarify in later functional studies whether Alien could substitute the regulatory activity of the pocket proteins concerning E2Fs.

Besides the identification of these E2F protein interactions with Alien, interestingly, additional interacting proteins were detected *in vivo* that are participating in cell cycle regulation. Hereby, for the first time, an interaction between p107 and Alien was shown. p107 is a member of the pocket protein family and is involved in regulation of the E2F transcription factors, namely E2F-4 and E2F-5.[5] A recent study showed an additional interaction of p107 with E2F-1.[34] We confirmed this interaction with our techniques and have indications that this complex is not very abundant and is described as only transiently formed

at specific times during the cell cycle. Interestingly, we also observed the interaction of Alien with pRB itself. Notably, a number of Alien-interacting protein components described here have also been described as interacting proteins of the pRB. CDK2, which is presented here for the first time as an interacting partner of Alien *in vivo*, phosphorylates pRB in a cell cycle-dependent manner, which is important for cells to progress through the cell cycle.[35] Hyperphosphorylation of pRB results in loss of interaction with E2F1 as well as the repression of transcriptional activity mediated by E2F-1 will be abrogated.[7] Whether CDK2 also phosphorylates Alien will be an interesting future experiments. Taken together, Alien interacts with pRB and shares a number of pRB interacting proteins with pRB, which implies that Alien could either serve as a platform to modulate these specific interactions or influences the function of these protein complexes although it might substitute the regulatory activity of the pocket proteins concerning the E2F activities.

Thus, it is conceivable that a multicomponent protein complex or a number of different protein complexes exist in parallel that are involved in the regulation of particular cell cycle processes with Alien as a central factor. Further studies are demanded to understand the spatial and temporal regulation of such protein complexes.

The analyzed protein complexes involving particular cell cycle regulatory proteins are shown here for the first time. Also using the presented approach composed of mass spectrometry and immunological techniques novel and previously described *in vivo* interactions of endogenously expressed proteins were detected, which suggests that this approach is a promising procedure to get insight in biological processes by analysis of protein interactions.

Abbreviations: CoIP, co-immunoprecipitation; IDM, interaction discovery mapping; PMF, peptide mass fingerprints; SELDI, surface-enhanced laser desorption/ionization; MS, mass spectrometry.

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Supporting Information Available: Supplemental Figures 1−5. This material is available free of charge via the Internet at http://pubs.acs.org.

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3 Diskussion

Die Identifizierung von Biomarkern oder molekularen Mechanismen, die entscheidend an der Tumorentstehung und Progression beteiligt sind, sind von großem medizinischen und biologischen Interesse. Bisher gibt es nur für wenig Tumortypen relevante Biomarker, welche eine frühzeitige Erkennung ermöglichen. Die routinemäßig eingesetzten Marker wie z. B. das 1994 erstmals beschriebene PSA (Nogueira et al., 2009), oder *cancer antigen*s (CA) wie CA19.9 beim z. B. gastrointestinalen Karzinom (von Kleist, 1986) bzw. CA125 beim Ovarialkarzinom (Canney et al., 1984) zeigen jedoch eine geringe Sensitivität und Spezifität. Für eine bessere Vorsorgeuntersuchung oder Verlaufskontrolle nach erfolgreicher Tumorresektion sind deshalb Biomarker mit höherer Sensitivität und Spezifität erforderlich. Es gibt des Weiteren keine geeigneten Marker, welche eine genaue Tumorstadieneinteilung und somit die Detektion im Frühstadium der Tumorentstehung erlauben.

Die vorliegende Arbeit beschäftigt sich mit der Detektion, Identifizierung und Charakterisierung von tumorrelevanten Proteinen in Serum, Zellen und Gewebe. Sie zeigt Wege auf, mit denen erfolgreich, mit hoher Sensitivität und Spezifität, proteomische Marker, u. a. durch die Etablierung neuer Methoden, identifiziert werden können. Bei der für die Onkologie wichtigen Charakterisierung tumorrelevanter Proteine standen jedoch nicht nur die identifizierten Marker im Vordergrund. Es wurde zudem der Transkriptionsfaktor E2F, welcher in Kooperation mit DP1/2 bzw. dem Tumorsupressor pRb eine entscheidende Barriere in der Tumorentstehung bildet, genauer untersucht. Im Fokus der Charakterisierung sowohl von Markern, als auch E2F standen dabei die Protein-Protein-Interaktionsstudien. Das Ziel dieser Studien ist es, die Biologie der Tumoren bzw. der entarteten zellulären Proliferation verständlicher zu machen und mögliche neue Ansatzpunkte für Medikamente in der Krebstherapie zu liefern.

3.1 Biomarkersuche in Serum und Zellen von CTCL Patienten

Serum hat ebenso wie Urin, Stuhl oder Speichel den Vorteil, als Ausgangsmaterial für die Biomarkersuche nicht bzw. nur minimal invasiv in großen Mengen gewonnen werden zu können. Der große Nachteil besteht jedoch darin, dass Serum Proteine mit einem Konzentrationsbereich von wenigen pmol/L bis zu hohen µmol/L (z. B. Albumin, 0,6

mmol/L) aufweist (Service, 2003) und somit neue Marker nur schwer zu identifizieren sind.

Das untersuchte Serum in der vorliegenden Arbeit wurde von Patienten mit Mycosis Fungoides (MF) einer Untergruppe des kutanen T-Zell-Lymphoms (CTCL) gewonnen. Diese, zur Gruppe der Non-Hodgkin-Lymphom zählende Krankheit, ist durch die Migration aktiver, veränderter T-Zellen in die Haut charakterisiert (Bagot, 2008). Für CTCL sind zurzeit nur wenig Marker bekannt. Bisher sind Serummarker wie Neopterin, der lösliche IL2-Rezeptor, β2-Mikroglobulin oder lösliche intrazelluläre Adhesionsmoleküle als Marker für Patienten mit CTCL postuliert (Hassel et al., 2004; López-Lerma et al., 2009). Aber auch diese Marker zeigen eine geringe Sensitivität und Spezifität.

Die Suche nach neuen Serummarkern mit hoher Sensitivität und Spezifität erfolgt in der vorliegenden Arbeit mit der *surface enhanced laser desorption/ionisation time of flight mass spectrometry* (SELDI-TOF-MS). Dabei wurden die Seren von 25 Patienten mit Mycosis Fungoides (MF) gegen 25 Seren gesunder Kontrollpersonen nach Applikation auf ProteinChip arrays massenspektrometrisch vermessen. Die anschliessende bioinformatorische Auswertung zeigte 8 potentielle Marker, welche die Kontrollgruppe von den MF Patienten mit einer Sensitivität von 82,6% und einer Spezifität von 100% separieren. Die Identifizierung der potentiellen Marker erfolgte mittels 2D-Gelelektrophorese und anschliessendem tryptischen Verdau. Das in MF herunterregulierte Protein mit einem Molekulargewicht von 13,7 kDa wurde als Transthyretin (TTR) identifiziert. Um zu zeigen, dass TTR dem differentiell exprimierten Protein mit einer Masse von 13,7 kDa entspricht, wurde eine Immundepletion durchgeführt. Hierbei wurde jedoch nicht nur das Signal bei 13,7 kDa depletiert, sondern drei weitere Signale mit ähnlichem Molekulargewicht. Bei diesen drei Signalen handelt es sich um posttranslationale Modifikationen von TTR. Alle drei Modifikationen, die cysteinylierte Form (13,8 kDa), die cystein/glycinylierte Form (13,9 kDa) und die gluthationylierte Form (14,1 kDa), waren ebenfalls deutlich gegenüber der Kontrollgruppe herabreguliert und tragen einen entscheidenden Anteil an der hohen Sensitivität und Spezifität bei. Ein *enzyme linked immunosorbent assay* (ELISA) konnte TTR als potentiellen Marker bestätigen. TTR als Einzelmarker zeigte dabei eine Sensitivität von 80,8% und eine Spezifität von 92%.Transthyretin ist ein in der Leber, Retina, Pankreas oder Hirnflüssigkeit exprimiertes Protein (Ando, 2009) und besitzt als Marker eine geringe Tumorspezifität. Es wurde in einigen Krankheiten wie z. B.

Ovarialkarzinom (Zhang et al., 2004), Lungenkrebs (Li et al., 2009) oder Oesophaguskrebs (Guerra et al., 2009) herunterreguliert, aber kaum in Krankheiten hochreguliert gefunden (Grus et al., 2008).

Beim Vergleich meiner Studie mit den Untersuchungen an Ovarialkarzinomen von Zhang et al. (Zhang et al., 2004), bzw. Kozak et al. (Kozak et al., 2005) sind klare Unterschiede in den Proteinexpressionsmustern zu erkennen. So konnte in diesen Studien neben der Herunterregulation von TTR bei Kozak et al. auch eine erhöhte Expression von α und β Hämoglobin nachgewiesen werden, was in meinen Untersuchungen mit MF Serum nicht nachgewiesen wurde. Dieser Unterschied lässt sich durch die erhöhte Blutversorgung epithelialer Tumore wie z. B. dem Ovarialkarzinom gegenüber dem Lymphom erklären. Des Weiteren konnte in meiner Studie nicht die verkürzte Form von TTR mit einem Molekulargewicht von 12,8 kDa nachgewiesen werden. Die Studie von Fung et al. (Fung et al., 2005), in der Brust- und Darmkrebs bzw. Prostata- und Ovarialkarzinome u. a. im Hinblick auf die TTR Expression im Serum untersucht wurden, konnte im Gegensatz zu meiner Studie keine Herunterregulation der cystein/glycinylierten TTR Modifikation nachweisen. Die Vergleiche mit anderen Studien zeigen TTR zwar als unspezifischen Tumormarker, seine posttranslationalen Modifikationen sind jedoch für MF als spezifisch anzusehen.

Aus diesen Ergebnissen lässt sich ableiten, dass die Unterschiede zwischen den einzelnen Erkrankungen in den Proteinmodifikationen oder bei gering konzentrierten Proteinen zu suchen sind. Die Identifikation neuer Serummarker setzt daher Fraktionierungstechniken wie z. B. Proteinanreicherung mittels Proteominer (Bandow, 2010) der Serumproben voraus.

Um CTCL genauer charakterisieren zu können, wurden auch die CD4+ T-Lymphozyten. in Kapitel 2 untersucht. Dafür wurde Blut von Patienten mit MF anhand eines Biocoll Dichtegradienten fraktioniert. Die Fraktion der mononukleären Zellen, welche auch die CD4+ T-Lymphozyten beinhaltet, wurde vorsichtig entnommen. Die weitere Aufreinigung der CD4+ T-Zellen erfolgte mittels *magnetic activated cell sorting* (MACS). Durch die Verwendung von CD4+ Antikörpern entstand so eine CD4+ T-Zell Fraktion und eine Durchflußfraktion, welche als CD4- bezeichnet wurde. Da sich CTCL durch die Migration aktiver CD4+ T-Zellen in die Haut charakterisieren lässt, war eine Suche nach Biomarkern erst durch die PHA-Aktivierung der CD4+ T-Zellen der Kontrollgruppe möglich. Für die Biomarkersuche wurden nun die CD4+ T-Zellen der CTCL Patienten mit den PHA aktivierten CD4+ T-Zellen der Kontrollgruppe verglichen,

gleiches galt für die CD4- Zellen. Die gewonnenen Proteine dieser Gruppen wurden massenspektrometrisch unter der Verwendung von H4 ProteinChips und der SELDI Technologie vermessen. Die Daten wurden anschliessend bioinformatorisch analysiert und beide Gruppen konnten mit großer Sensitivität und Spezifität voneinander unterschieden werden. Die CD4+ Gruppe konnte mit einer Sensitivität von 84% und einer Spezifität von 91,7% von der Kontrollgruppe separiert werden, die CD4- Gruppe mit 95,8% bzw. 96%. Die bessere Separierung der CD4- Gruppe von der Kontrolle lässt sich dadurch erklären, dass in der CD4+ Fraktion nur CD4+ T-Zellen enthalten sind und die CD4- Fraktion aus verschiedenen Zellen wie z. B. Granulozyten oder CD8 positiven Lymphozyten besteht. Diese liefern eine größere Proteinvielfalt, über die jedoch bisher wenig im Zusammenhang mit CTCL bekannt ist.

Die beschriebene Methode hat den Vorteil gegenüber anderen Untersuchungen wie cDNA arrays von Tracey et al. (Tracey et al., 2003) oder Serumuntersuchungen von Hassel et al. (Hassel et al., 2004) dass mit ihr proteomische Untersuchungen direkt am Ausgangsort von CTCL, den CD4+ T-Zellen, nach neuen Markern gesucht wird. Diese Methode hat jedoch den Nachteil, relativ zeitaufwendig gegenüber der einfachen Applikation von Serum auf einen ProteinChip zu sein. Dazu kommt, dass nach der Lyse der aufgereinigten CD4+ Zellen zwar genug Proteine für eine massenspektrometrische Analyse vorhanden sind, jedoch kaum für folgende Identifikationsversuche. Daher konnte in dieser Studie nur HNP3 aufgrund seines typischen massenspektrometrischen Profils im Verbund mit HNP1 und HNP2 mittels Immundepletion identifiziert werden.

HNP3 gehört zur Gruppe der Defensine, welche den antimikrobiellen Peptiden zuzuordnen sind. Antimikrobielle Peptide haben ihre Aufgabe in verschiedenen Aspekten der Immunität (Entzündung, Wundheilung, Regulation des adaptiven Immunsystems) und der Homöostase (Auvynet et al., 2009)**.** Humane Defensine sind unterteilt in α und β Defensine. Sie sind kationische, Tridisulfid-Peptide und werden in epithelialen Zellen (Cunliffe et al., 2002) oder bestimmen Leukozyten wie den neutrophilen Zellen exprimiert (Selsted et al., 2005). Beim Menschen sind 6 verschiedene α Defensine, HNP 1-4 in neutrophilen Zellen und HD5 / HD6 in den Paneth Zellen bekannt (Droin et al., 2009). Eine Disregulation von α oder β Defensinen sind in einer Vielzahl von Tumoren wie z. B. dem Nierenzellkarzinom (Müller et al., 2002), dem Plattenepithelkarzinom der Zunge (Lundy et al., 2004) oder dem Kolerektalkarzinom (Melle et al., 2005) beschrieben. Des Weiteren spielen Defensine eine Rolle in der adaptiven Immunantwort, indem sie chemotaktisch auf T-Zellen wirken

(Chertov et al., 1996). Diese Untersuchungen zeigen, dass Defensine neben ihren antimikrobiellen Eigenschaften eine wichtige und bisher noch unklare Rolle in der Tumorbiologie spielen. Jedoch ist dies die erste Arbeit, in der Defensine in einen Zusammenhang mit CTCL, vor allem als Biomarker, gebracht werden. Die Identifizierung der anderen differentiell exprimierten Proteine in CD4- und CD4+, insbesondere des Proteins mit dem Molekulargewicht von 8565 Da, welches auch in Serumuntersuchungen von CTCL Patienten differentiell exprimiert zur Kontrollgruppe gefunden wurde, soll das Verständnis der Biologie des CTCL verbessern helfen. Als mögliche Proteine für das Signal mit 8565 Da sind Interleukin 8 (Chain: 27-99, pI: 9.24, Mw: 8542, expasy.org), welches chemotaktisch auf neutrophile und basophile Zellen bzw. T-Zellen wirkt, und Apolipoprotein A2 zu nennen (UniProtKB/Swiss-Prot P02652; Chain: 24-99, Mw: 8580). Apolipoproteine werden häufig disreguliert in Serum von Patienten mit Tumoren (Vermaat et al., 2009; Nossov et al., 2008; Yi et al., 2009) detektiert. Eine Aufgabe von Apolipoprotein A2, welches ebenso wie TTR in der Leber gebildet und in das Serum sezerniert wird, ist der reverse Cholesterintransport (Gillard et al., 2009). Die Rolle von Apolipoproteinen in der Tumorentstehung oder Karzinogenese ist jedoch ungeklärt.

3.2 Detektion und Identifizierung von Markerkandidaten in Gewebe und Stroma

Die vom Tumor abgegebenen Markerkandidaten verlieren in der Peripherie des Körpers stark an Konzentration gegenüber dem Ort ihrer Sekretion. Aus diesem Grund ist es sinnvoller, Biomarker nicht in der Peripherie, wie z. B. im Serum oder Urin zu suchen, sondern direkt im Tumorgewebe. Hierbei ist aber zu beachten, dass das resektomierte Material oft nicht nur aus Tumorgewebe, sondern auch aus Normalgewebe oder Nekrosen bestehen kann. Um falsch positive Marker aus gesundem oder nekrotischem Gewebe zu umgehen, ist eine Mikrodissektion des Tumormaterials unabdingbar.

Die für die Studie in Kapitel 3 verwendeten Tumorproben stammen aus dem Pankreas und wurden alle vor der Massenspektrometrie mittels SELDI pathologisch untersucht. Tumorzellen (3000-5000) wurden dabei mikroseziert, die Proteine nach der Zelllyse massenspektrometrisch vermessen und in einem 2D-Gel elektrophoretisch aufgetrennt. Von den so separierten Proteinen wurden ca. 95 Proben ausgeschnitten und tryptisch verdaut. Insgesamt konnten so 29 Proteine identifiziert werden, von denen zwei mit den

bioinformatisch ermittelten, differentiell exprimierten Proteinen korellierten. Diese beiden Proteine konnten als DJ-1 und HSP27, welche im Pankreskarzinom gegenüber der Kontrollgruppe heraufreguliert gefunden worden, identifiziert werden. Während HSP27 nur zwischen zentralem Pankreaskarzinom, Karzinomrand und der gesunden Kontrollgruppe differenziert, separierte DJ-1 zentrales Pankreaskarzinomgewebe vom Tumorrand, dem angrenzenden gesunden Pankreasgewebe und der gesunden Kontrollgruppe. Die Zugehörigkeit der beiden Proteine zu den massenspektrometrisch ermittelten Signalen wurde u. a. mittels Immundepletion nachgewiesen. Die Immunhistochemie konnte mittels spezifischen Antikörpern gegen HSP27 und DJ-1 die Daten der Massenspektrometrie ebenfalls bestätigen. Ausserdem konnte mittels ELISA auch HSP27 im Serum von Pankreaskarzinompatienten heraufreguliert gefunden werden, was ohne die Daten der Mikrodissektion nicht möglich gewesen wäre.

HSP27 ist ein Chaperon und hat Funktionen hinsichtlich der Stress Resistenz und Aktin Organisation. Ausserdem ist es in der Lage, mit einigen Komponenten der programmierten Zelltotmaschinerie zu interagieren. Die Lokalisation von HSP27 erstreckt sich vom Zytoplasma, den Nukleus, über das Zytoskelett bis hin zu den mitotischen Spindeln. Weiterhin ist bekannt, dass eine schlechte Prognose bei Knochenkrebs, Magen- und Lebertumoren mit HSP27 einhergeht (Garrido et al., 2003; Ciocca et al., 2005) Letztendlich scheint die Rolle von HSP27 in Pankreaskarzinomen aber immer noch unbekannt, denn bisherige Publikationen sind widersprüchlich (Gangarosa et al., 1999; Lu et al., 2004)

Das Protein DJ-1, welches ebenfalls im Pankreaskarzinom hochreguliert gefunden wurde, wird vom Gen PARK7 kodiert. Defekte in Park7 führen jedoch nicht nur zu Parkinson (Miller et al., 2003)**.** Weitere Funktionen sind im Bereich der zellulären Transformation, der Fertilisation beim Mann, oder ebenso wie HSP27 als Sensor für oxidativen Stress und Chaperonfunktion beschrieben (Nagakubo et al., 1997; Honbou et al., 2003; Shendelman et al., 2004). Eine Überexpression von PARK7 ist in Lungenkarzinomen oder Brustkrebs beschrieben, wobei PARK7 bei Brustkrebs negativen Einfluss auf den Tumorsuppressor PTEN nimmt, was eine Überexpression von hyperphosphoriliertem PKB / Akt zu Folge hat und letztendlich in einer höheren zellulären Lebenserwartung resultiert (Kim et al., 2005). In proteomischen Studien von Magenkrebs konnte DJ-1 nur im metastasierenden Gewebe und nicht im nicht metastasierenden Tumor bzw. gesunden Gewebe nachgewiesen werden (Chen et al., 2004).

Die im Kapitel 3 beschriebenen proteomischen Biomarker HSP27 und DJ-1 für Pankreaskarzinome wurden nach der Mikrodissektion von Tumorgewebe (zentraler Tumor und Tumorrand) und gesundem Kontrollgewebe identifiziert. Um jedoch weiter in funktionelle Bereiche der Tumore, das Stroma, und somit einer genaueren räumlichen Lokalisation der Biomarker vorzudringen, wurde die *tissue on chip* Massenspektrometrie (toc-MS) entwickelt. Denn es ist bekannt, dass die Interaktion zwischen Tumor und Stroma, insbesondere den Tumor assoziierenden Fibroblasten (TAF), Tumorendothelzellen (TEZ) und Tumor assoziierte Makrophagen (TAM) im Zusammenhang mit Tumorprogression stehen.

Für die Versuche in Kapitel 4 wurden Kopf-Hals-Tumore verwendet, da die wenigen, uns zur Verfügung stehenden Pankreaskarzinome kein ausgeprägtes Stroma aufwiesen. Um eine Übersicht über das resektomierte Gewebe zu bekommen, wurde eine Haematoxylin/Eosin-Färbung (HE) durchgeführt. Anschliessend wurde ungefärbtes Stroma aus dem vom Tumor umgebenem Stroma und gesunden Gewebe mittels PALM mikroseziert, die Areale unter dem Binokular auf einen ProteinChip appliziert, lysiert und anschliessend massenspektrometrisch vermessen. Mit dieser neu entwickelten Methode ist es nun erstmals möglich, 100-500 stromale Zellen massenspektrometrisch zu analysieren. Vorangegangene Studien (Melle et al., 2007) benötigten für eine Studie mit SELDI in Verbindung mit Mikrodissektion bis zu 5000 Zellen.

Die Analyse der massenspektrometrischen Daten ergab 13 differentiell exprimierte Signale, die zwischen Tumor und Normalstroma unterscheiden. Dabei konnten 8 Signale im Massenbereich von 2 kDa bis 20 kDa (*low*) und fünf im Bereich zwischen 20 kDa und 200 kDa (*high*) detektiert werden, welche beide Gruppen mit einer Sensitivität von bis zu 92,8% und einer Spezifität von 100% separieren. Das am besten differenzierende Signal in der low Gruppe hatte ein Molekulargewicht von 7477 Da und resultiert nach initialer Datenbanksuche in zwei möglichen Proteinen, beta defensin 119 (UniProtKB/Swiss-Prot Q8N690 Chain: 22-84, Mw: 7493 Da) und *fibroblast growth factor 23 C-terminal peptide* (UniProtKB/Swiss-Prot Q9GZV9 Chain: 180-251, Mw: 7520). Über die Expression von β Defensinen im Stroma gibt es bisher keine Informationen. Jedoch zeigen β Defensine, ebenso wie α Defensine, anitbakterielle Aktivität und sind nicht einheitlich in Tumoren exprimiert (Joly et al., 2009; Droin et al., 2009). Über das zweite Protein, den *fibroblast growth factor 23,* ist bisher wenig bekannt. Es scheint jedoch eine Rolle bei der Phosphat-Homöostase zu spielen (Ramon et al., 2010). Generell ist über Tumor assoziierende Fibroblasten bekannt, dass sie aufgrund ihrer räumlichen Nähe zu den

Tumorzellen in der Lage sind, Wachstumsfaktoren wie *transforming growth factor beta* (TGF beta) oder *vascular endothelial growth factors* (VEGF) direkt an den Tumor abzugeben und somit wachstumsfördernd auf Tumorzellen wirken (Hofmeister et al., 2008). Das physiologische Zusammenspiel von Tumor und Stroma erhärtet die Vermutung, dass das in dieser Arbeit überexprimiert gefundene Signal von 7477 Da dem *fibroblast growth factor 23* zugeordnet werden könnte. Für das Protein im hohen Bereich mit einem Molekulargewicht von 80 kDa ist als Markerkandidat das *Sci*-Onkogene (UniProtKB/Swiss-Prot P12755, Mw: 80,005 Da) zu nennen. Seine Funktion ist jedoch konträr beschrieben. Einerseits wurde seine Fähigkeit als Onkogen, indem es transformierend auf Hähnchenembryofibroblasten wirkt, publiziert. Auf der anderen Seite sind aber auch antitumorigene Eigenschaften, auch im Zusammenhang mit Metastasenbildung, von Ski beschrieben (Le Scolan et al., 2008).
Diese Arbeit zeigt nicht nur einen Weg zur protemischen Biomarkersuche im Stroma, sondern auch nützliche Ansätze, Metastasierungsmarker, welche sich ebenfalls im Stroma befinden können, zu detektieren und später auch zu identifizieren. Denn nicht nur der Primärtumor, sondern auch die Metastasen tragen entscheidend zur geringeren Lebenserwartung eines Patienten bei.

3.3 Charakterisierung tumorrelevanter Proteine

Proteine erfüllen ihre Funktion selten allein, sondern werden oft erst durch die Interaktion mit anderen Proteinen in einem Netzwerk funktionell aktiv. Aus diesem Grund ist die Identifizierung von Bindungspartnern für die Charakterisierung des physiologischen Protein Verbundes unabdingbar (Coulombe et al., 2004). Die in dieser Arbeit dargestellten Ergebnisse zeigen zu einem die Identifizierung von Protein-Protein-Interaktionspartnern von TTR, einem in Kapitel 1 identifizierten Serummarker für MF. Zum anderen wurde der Transkriptionsfaktor E2F1 genauer charakterisiert. Dieses Mitglied der E2F Transkriptionsfaktorfamile, welches im Verbund mit DP1/2 und pRb (Weinberg, 1995) eine entscheidende Barriere in der Tumorentstehung bildet, soll, im Gegensatz zu den Biomarkern, welche für eine Früherkennung von Tumoren essentiell sind, die zellulären Mechanismen der Tumore bzw. Tumorentstehung genauer verstehen helfen.
Für die Charakterisierung von TTR, dem Serummarker aus MF, wurde zum ersten mal die blue-native Polyacrylamidgelelektrophorese (BN-PAGE) (Camacho-Carvajal et al., 2004) modifiziert und mit der SELDI-Technik kombiniert (Kapitel 5). Des Weiteren

wurde die Hypothese aufgestellt, dass die Herunterregulation eines Proteins aus einem Proteinnetzwerk nicht ohne Folgen für seine Interaktionspartner hinsichtlich deren Expression sein kann. Aus diesem Grund schien die Identifizierung von TTR nicht nur hilfreich im Hinblick auf die physiologische Bedeutung dieses Proteins, sondern auch für die Suche nach neuen Serummarkern in MF. Der Vorteil der BN-PAGE liegt darin, dass keine teuren Antikörper für die Detektion von Interaktionspartnern benötigt werden. Des Weiteren können eben diese nicht benutzten Antikörper auch keine Bindungstellen für Protein Interaktionspartner besetzen. Ein Nachteil dieser Technik liegt jedoch darin, dass Proteinkomplexe mit dem gleichen Molekulargewicht in einer Bande zusammenlaufen und diese Interaktionen immer mit einer anderen Technik, wie in unserem Fall der Koimmunpräzipitation bestätigt werden müssen.

In meinerr Studie konnte Apolipoprotein A1, Apolipoprotein A4, das Retinol bindende Protein 4 (RBP-4) und der Retinoid X Rezeptor β (RXR-β) als TTR Interaktionspartner identifizieren werden. Um die aufgestellte Hypothese, dass Interaktionspartner eines Biomarkers ebenfalls Markerkandidaten sein können, zu bestätigen, wurden die molekularen Massen der vier Interaktionsparter mit den massenspektrometrischen Daten verglichen. Hierbei korrelierte die molekulare Masse von Apolipoprotein A1 mit einem massenspektrometrischen Signal. Eine Immundepletion konnte diese Zugehörigkeit von Apolipoprotein A1 zu dem massenspektrometrischen Signal beweisen.

Während TTR in Zellen der Retina (Getz et al., 1999), dem Plexus choroideus (Herbert et al., 1986) oder der Leber (Prapunpoj et al., 2009) exprimiert wird, wird Apolipoprotein A1 u. a. in der Leber und im Dünndarm (Zhou et al., 2009) exprimiert. Beide Proteine werden in das Serum sezerniert. Eine Interaktion beider Proteine wurde im Plasma von Sousa et al. (Sousa et al., 2000) beschrieben. Während Transthyretin in Tumoren oder anderen Krankheiten herauf- oder herunterreguliert gefunden wurde (Escher et al., 2007; Biroccio et al., 2006; Grus et al., 2008), wurde Apolipoprotein A1 herunterreguliert in Ovarialkarzinomen oder Schizophreniepatienten gefunden (Zhang et al., 2004; La et al., 2007). Isoformen von Apolipoprotein A1 konnten aber auch in der cerebrospinalen Flüssigkeit von Parkinson Patienten hochreguliert gefunden werden (Wang et al., 2010).

Das überraschendste Ergebnis dieser TTR Charakterisierung war jedoch die Präsenz und Interaktion von TTR zu RXR-β im Serum. Retinoide wie Retinoid acid Rezeptoren (RAR) und Retinoid X Rezeptoren (RXR) inhibieren die Proliferation und induzieren die Differenzierung in Melanomzellen (Chakravarti et al., 2007). Nukleare Retinoid

Rezeptoren sind ligandenabhängige Transkriptionsfaktoren. Abnormale Expression bzw. Funktion von RAR oder RXR spielen eine entscheidende Rolle beim Wachstum verschiedener Tumore (Kanamori et al., 2007). Für die Behandlung von CTCL ist 4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)ethynyl] benzoic acid, oder besser bekannt als das Retinoid Bexarotene, weit verbreitet (Wagner et al., 2009). Klinische in vitro Studien an T-Zell-Leukemie zeigen nach Bexarotenbehandlung einen Verlust des RXR α Rezeptors (Lin et al., 2008). Bereits 1996 beschrieb Bellovino et al. (Bellovino et al., 1996) die Sekretion des TTR-RBP Komplexes aus der humanen Leberzellkarzinomzelllinie HepG2. Ähnliche Mechanismen könnten für TTR im Komplex mit RXR-β gelten, was das Vorkommen von RXR im Serum erklären könnte. Eine andere Erklärung könnte sein, dass nach dem Zelltod Fragmente von RXR-β ins Serum gelangten und diese noch fähig zur Komplexbildung u. a. mit RBP und TTR sind. Die Liganden der Retinoidrezeptoren, die Retinoide, umfassen eine zum Vitamin A (Retinol) analoge Gruppe. Das Retinol bindende Protein (RBP) ist das spezifische Transportprotein für Vitamin A und transportiert dieses von der Leber in das Zielgewebe. Die Interaktion von RBP4 und TTR, die in meiner Studie zum ersten Mal in CTCL beschrieben wurde, konnte Soares et al. (Soares et al., 2005) im Zusammenhang mit amyloider Neurophaty bringen.

Zusammenfassend lässt sich sagen, dass die ungeklärte Rolle von TTR bei Tumoren mit der Interaktion zu RXR-β im Serum einen Übergang vom allgemeinen Tumormarker hin zum besseren Verständnis der Progression von Tumoren bilden könnte. Aus diesem Grund können Veränderungen im Expressionslevel von TTR und dessen Interaktinospartnern, welche für die Bindung von Retinoiden an RAR oder RXR verantwortlich sind, Konsequenzen für die Zellproliferation in CTCL haben, was wiederum durch das Medikament Bexarotene behandelt werden kann (Querfeld et al., 2006).

In der Krebstherapie spielt jedoch nicht nur die Identifizierung von Biomarkern für die Tumorfrüherkennung eine wichtige Rolle, sondern auch das biologische Verständnis über zelluläre Prozesse wie Zell-Differenzierung oder Proliferation. Denn so ist es möglich, neue Ansatzpunkte in der Therapie gegen Tumorerkrankungen zu entwickeln. In der vorliegenden Arbeit wurde deshalb der Transkriptionsfaktor E2F1, welcher im Verbund mit den *differentiation regulated transcription factor-binding proteins* (DP) und dem Tumorsupressor pRb eine der wichtigsten zellulären Barrieren in der Krebsentstehung bildet, mittels Protein-Protein-Interaktionsstudien genauer in Kapitel 6 charakterisiert.

Die hierbei verwendete Methode zur Identifizierung von Protein-Protein-Interaktionen wurde von mir in meiner Diplomarbeit etabliert und erstmals im Jahr 2005 von Lehmann et al. (Lehmann et al., 2005) publiziert. Im Gegensatz zum Hefe-Zwei-Hybrid-System, welches *in vitro* mit hoch exprimierten Proteinen arbeitet, aber oft zu falsch positiven Ergebnissen führt, ist diese Methode in der Lage, mittels Antikörperbindung an ein Trägermaterial (IDM beads) endogene Proteine zu präzipitieren und die einzelnen Proteine des Proteinkomplexes dann mittels SELDI massenspektrometrisch zu vermessen. Die anschliessende Identifikation mittels Peptid-Massen-Fingerabdruck und anschliessender Validierung durch eine Koimmunpräzipitation verringert die Detektion falsch positiver Ergebnisse um ein Vielfaches. Mit dieser hoch sensitiven Methode wurde E2F1 in der Zelllinie U2OS genauer charakterisiert. Dabei konnte ein neuer Interaktionspartner mit einem Molekulargewicht von ca. 36 kDa massenspektrometrisch detektiert und anschliessend als Protein Alien identifiziert werden. Diese Interaktion beider Proteine konnte einerseits mit einem reziproken Immunassy, d. h. Alien Antikörper wurden an die IDM beads gebunden und endogenes E2F1 als Interaktionspartner nachgewiesen, bzw. in GST pull-down Versuchen bestätigt werden. Funktionelle Analysen konnten den reprimierenden Einfluss von Alien auf E2F1 deutlich darlegen. Untersuchungen in Kooperation mit Tenbaum et al. (Tenbaum et al., 2007) charakterisieren diese Interaktion in Folgeversuchen und zeigen die unterschiedliche Expression von Alien in verschiedenen Zellzyklusstadien. Neben dieser Interaktion konnte im Massenspektrometer aber auch die Interaktion zwischen E2F3 und Alien nachgewiesen werden. Bei der Koimmunpräzipitation zur Bestätigung dieses Ergebnisses wurde auch die Rolle von Alien zu anderen Transkriptionsfaktoren der E2F-Familie untersucht. Hierbei ergaben sich weitere Interaktionen von Alien zu Mitgliedern der E2F-Famile und legten damit eine zentrale Rolle von Alien in der Zellzyklusregulation dar. Weiterführende Experimente konnten Interaktionen von Alien mit pRb, p107 und CDK2 nachweisen. Diese neuen Alien - Interaktionspartner wurden mittels Koimmunpräzipitation validiert, wobei die Interaktion von Alien mit pRb zuätzlich durch das Hefe-Zwei-Hybrid-System bestätigt werden konnte. Hier konnte gezeigt werden, das Alien in den Bereich der intakten pocket Domäne bindet, wohingegen die Interaktion von Alien mit der pRb Mutante pRb706, in der ein Aminosäureaustausch in der pocket Domäne detektierbar ist, nicht nachzuweisen war.

Diese Ergebnisse zeigen die wichtige Rolle von Alien in der Zellzyklusregulation, indem es tief im Netzwerk mit Mitgliedern der E2F Transkriptionsfamilie und Mitgliedern der

pocket Proteinfamilie verankert ist. Bisherige Publikationen zeigten den Einfluß von Alien als Corepressor auf nukleare Hormonrezeptoren wie dem Vitamin D3 Rezeptor und dem Thyroidhormon Rezeptor (Polly et al., 2000). Corepressoren sind in der Lage, ohne direkte Bindung an die DNA die Transkription zu inhibieren. Diese Aufgabe der Repression für E2F1-4 wurde bisher von pRb postuliert (Trimarchi et al., 2002). Die in unserer Arbeit verwendete Cervixkarzinom-Zelllinie C33A ist bekannt dafür, kein funktionell aktives pRb zu exprimieren (Scheffner et al., 1991). Daraus lässt sich schließen, das Alien seine repremierende Wirkung auf E2F1 unabhängig von pRb ausübt. Ähnliche Mechanismen konnten für Prohibitin, HP1-γ oder Max (Frolov et al., 2004) in Verbindung mit E2F1 nachgewiesen werden. Des Weiteren weisen E2F1 und Alien weitere gemeinsame Bindungspartner, wie p107 (Escher et al., 2007; Calbó et al., 2002), Sin3A und HDAC (Dressel et al., 1999; Moehren et al., 2004; Lai et al., 2001) auf, was widerum die Funktion von Alien im Proteinnetzwerk der Zellzyklusregulation festigt. Diese Ergebnisse lassen vermuten, dass ein großer Multiproteinkomplex oder mehrere kleine Komplexe mit Alien als eines der zentralen Proteine in die Regulation des Zellzyklus eingreifen. Die Interaktion von Alien zu CDK2, welches die Phosphorylierung von pRb zellzyklusabhängig übernimmt (Akiyama et al., 1992), wurde hier zum ersten mal beschrieben. CDK2 hyperphosphoryliert pRb, was zum Bindungsverlust von E2F1 führt und somit wieder die transkriptionelle Aktivität von E2F1 herbeiführt (Helin et al., 1993). Ob CDK2 auch die Funktion der Phosphorylierung von Alien übernimmt, bleibt aber weiterhin unklar.

Zusammenfassend lässt sich sagen, das die Charakterisierung des Transkriptionsfaktors E2F1 neue Interaktionsparter hervorgebracht hat, die im Falle von Alien nicht nur auf E2F1 reprimierenden Einfluß hat, sondern auch eine entscheidende Rolle in der Zellzyklusregulation spielen.

Zusammenfassend lässt sich sagen, dass in der vorliegende Arbeit Markerkanditaten mit hoher Sensitivität und Spezifität sowohl im Serum, Zellen als auch im Gewebe detektiert und identifiziert wurden. Des Weiteren konnten neue Methoden wie die toc-MS entwickelt, bzw. die Kombination von SELDI mit BN-PAGE zur Charakterisierung von Biomarkern in CTCL bzw. Nachweis neuer Markerkandidaten etabliert werden. Die proteomische Charakterisierung von E2F1 zeigte eine Interaktion mit dem Protein Alien. Alien wirkt reprimierend auf E2F1 und ist ein neues und wichtiges Mitglied in der Zellzyklusregulation und liefert bei dem Verständnis der Tumorproliferation neue molekularbiologische Einblicke.

4 Zusammenfassung

Biomarker sind für die rechtzeitige Erkennung von Tumorerkrankungen von großer Bedeutung. Jedoch weisen die bisher verwendeten Biomarker wie z. B. das prostataspezifische Antigen (PSA) beim Prostatakarzinom oder *cancer antigens* (CA) wie z. B. CA19.9 bei gastrointestinalen Tumoren eine relativ geringe Sensitivität und Spezifität auf. Aus diesem Grund ist es notwendig, neue Marker zu identifizieren bzw. neue Methoden zur Detektion für die Biomarkersuche zu entwickeln, welche dann eine bessere Vorsorge bzw. rechtzeitige Behandlung und damit eine erhöhte Lebenserwartung ermöglichen könnten. Neben der Identifikation neuer Biomarker ist aber auch die Identifizierung derer Interaktionspartner für ein besseres biologisches Verständnis der entarteten zellulären Proliferation erforderlich. Somit könnten dann auch neue Ansatzpunkte für Medikamente im Kampf gegen den Krebs gefunden werden.

Diese Arbeit soll Möglichkeiten aufzeigen, Biomarker aus Serum, Gewebe oder einzelnen Zellen zu detektieren und zu identifizieren. Die Charakterisierung dieser Biomarker soll dann deren Einordnung in bestehende Signalwege erlauben und die molekularen Mechanismen sowie die biologischen Funktionen besser verstehen helfen.

Die Detektion neuer proteomischer Biomarker und neuer regulatorischer Mechanismen der Proliferation erfolgte mittels *surface enhanced laser desorption/ionisation time of flight mass spectrometry* (SELDI-TOF-MS). Für die Biomarkersuche im Serum wurden die massenspektrometrisch generierten Spektren von Patienten mit kutanem T-Zell-Lymphom (CTCL) gegen eine gesunde Kontrollgruppe verglichen. Als Ergebnis konnten hierbei 8 Signale die CTCL-Gruppe von der gesunden Kontrollgruppe mit einer Sensitivität von 82,6% und einer Spezifität von 100% separieren. Mittels 2D-Geleletrophorese, tryptischem Verdau und Immundepletion konnte das Protein Transthyretin (TTR) und drei posttranslationale TTR-Modifikationen, welche im Vergleich zur Kontrollgruppe stark herunterreguliert sind, identifiziert werden.

Für die Detektion und Identifizierung neuer Marker aus Zellen wurden mittels magnetisch aktivierter Zell Separation (MACS) CD4+ und CD4- Lymphozyten aus CTCL-Patientenblut gewonnen. Nach massenspektrometrischer Vermessung konnte eine Vielzahl von Markern detektiert werden, die die gesunde Gruppe von den Patienten mit mehr als 90% in Sensitivität und Spezifität voneinander unterscheiden. HNP3 wurde dabei als potentieller Marker identifiziert.

Für die Biomarkersuche im Gewebe wurden massenspektrometrisch generierte Spektren von mikrosezierten Pankreastumoren analysiert. Als Markerkandidat konnte das im Tumorgewebe hochregulierte HSP27 erstmals identifiziert werden und anschliessend mittels ELISA ebenfalls im Serum als potentieller Marker nachgewiesen werden.

Um auch innerhalb eines Gewebes die Biomarker besser funktionalen Bereichen zuordnen zu können, wurde eine räumlich hochauflösende, massenspektrometrische Technik entwickelt. Mit der *tissue on chip* Massenspektrometrie (toc-MS), war es dann erstmals möglich, 100-500 stromale Zellen aus Kopf-Hals-Tumoren massenspektrometrisch zu vermessen und Tumorstroma vom Normalgewebsstroma proteomisch mit einer Sensitivität von 92.8% und eine Spezifität von 100% zu unterscheiden. Als potentielle Markerkandidaten konnten dabei das *fibroblast growth factor 23 C-terminal peptide* und das *ski oncogene* identifiziert werden.

Für die Onkologie ist jedoch nicht nur die Detektion und Identifizierung von tumorrelevanten Proteinen von großem Interesse, sondern auch die Einordnung in das biologische Umfeld. Dies geschieht durch die Charakterisierung der Proteine mittels Interaktionsstudien. In der vorliegenden Arbeit wurden nicht nur diese neu gefundenen Biomarker charakterisiert, sondern auch Proteine aus Signalwegen, die bekanntermaßen eine entscheidende Rolle für die Proliferation der Zelle spielen. Die Wahl fiel hier auf den Transkriptionsfaktor E2F1, welcher im Verbund mit den *differentiation regulated transcription factor-binding proteins* (DP) und dem Tumorsupressor pRb eine der wichtigsten zellulären Barrieren in der Krebsentstehung bildet. Die Zelllinien U2OS und C33A wurden für Immunpräzipitationsversuche verwendet. Dabei wurde Alien als neuer E2F1 Interaktionspartner gefunden. Bei der Charakterisierung der E2F1-Alien Interaktion konnte eine funktionell reprimierende Wirkung von Alien auf E2F1 nachgewiesen werden.

Die Charakterisierung des in CTCL gefundenen Biomarkers TTR erfolgte mit einer modifizierten *blue-native* Polyacrylamidgelelektrophorese (BN-PAGE). Hierbei konnte Apolipoprotein A1, Apolipoprotein A4, Retinol bindendes Protein *4* (RBP-4), und Retinoid X Rezeptor β (RXR-β) detektiert und als TTR Interaktionspartner identifiziert werden. Zusätzlich konnte einer der Interaktoren, das Protein Apolipoprotein A1, im Serum von CTCL Patienten herunterreguliert gefunden werden. Mit der Identifizierung von RBP-4 und RXR-β als TTR Interaktoren wurde eine Verbindung zwischen dem allgemeinen Tumormarker TTR und der Zellproliferation geschaffen.

Die Arbeit zeigt, dass die Suche nach Biomarkern mit entsprechenden Methoden erfolgreich in Serum, Zellen und Gewebe durchgeführt werden kann. Diese Marker bedürfen in einem weiteren Schritt einer biologischen Charakterisierung, die dann neben klinischen Studien eine Validierung dieser neu gefunden Marker erlaubt und Ansatzpunkt für neue Medikamente sein kann.

5 Synopsis

Biomarkers have an important impact in duly detection of tumours. Nevertheless, recent biomarkers like prostate specific antigen (PSA) for prostate cancer or cancer antigens (CA) like CA19.9 for gastrointestinal cancer lack of sensitivity and specificity. Therefore, it is essential to identify new biomarkers or to develop new techniques that lead to the detection and identification of new biomarkers which facilitate a much better precaution and early cancer treatment. Besides the identification of biomarkers it is essential to identify interaction partners of tumour relevant proteins at the transcriptional level. These interactors will help to get a better understanding of tumour proliferation on the one hand and might lead to the development of new medicaments in cancer treatment on the other hand.

This work shows strategies of biomarker detection and identification in serum, tissue and cells. Further, the characterisation of these tumour relevant proteins via protein-protein interaction studies is necessary to allow the grading in known pathways and molecular mechanisms for a better functional understanding.

The detection of both, new proteomic biomarkers and interaction partners of transcriptional regulatory proteins were performed with the surface enhanced laser desorption/ionisation time of flight mass spectrometry (SELDI-TOF-MS).

Biomarker discovery in serum was performed with SELDI generated spectra of patients with cutaneous T-cell lymphoma (CTCL) and compared with a healthy control group. Hereby, 8 signals separate both groups very well with a sensitivity of 82.6% and a specificity of 100%. With a technical combination of 2D-gelelectrophoresis, tryptic digestion and immunodepletion we identified transthyretin (TTE) and three of its posttranslational modifications significantly down regulated in the CTCL group.

Further, to detect biomarkers cells, CD4+ and CD4- lymphocytes were separated from whole blood samples of CTCL patients using magnetic activated cell separation (MACS) after biocoll density gradients. Bioinformatic analysis of the data generated by mass spectrometry revealed several marker candidates that separate the healthy control group from patients with a sensitivity and specificity of more than 90%. Hereby, HNP3 was identified as a potential marker.

Microdissected pancreas carcinomas were used to detect biomarkers in tissue. In this study we detected and identified HSP27 up regulated in cancer tissue compared to a

healthy control group and confirmed these findings even in serum using an enzyme linked immunosorbent assay (ELISA).

Further, a highly sensitive mass spectrometry named tissue on chip mass spectrometry (toc-MS) was developed to obtain a better biological resolution of the tissue. Though, it was possible to analyse 100-500 stromal cells from head and neck cancer and healthy control head and neck stromal tissue mass spectrometrically for the first time. Both, cancer stroma and control stroma could be separated with a sensitivity of 92.8% and a specificity of 100%. Further investigations revealed the fibroblast growth factor 23 C-terminal peptide and the ski oncogene as potential marker candidates.

Nevertheless, besides the detection and identification of tumour relevant proteins its grading into the biological environment has a big oncological impact. This characterisation of proteins can be done via protein-protein interaction studies. The present work does not only deal with the characterisation of biomarkers but also with cell proliferation and cell cycle regulating proteins. Thereto, we chose the transcription factor E2F1 which plays via its interaction to the differentiation regulated transcription factor-binding protein (DP) and pRb an essential role in cellular barrier against tumour appearance. Therefore, the cell lines U2OS and C33A were used for protein-protein interaction studies. Thereby, alien was found as a new E2F1 interaction partner. Further, the characterisation of alien shows a functional repression of E2F1.

The characterisation of the CTCL serum marker TTR was performed with a modified blue native polyacryleamide gelelectrophoresis (BN-PAGE). Apolipoprotein A1, apolipoprotein A4, retinol binding protein 4 (RBP-4), and fragments of the retinoid X receptor β (RXR-β) were found as TTR interacting proteins. Furthermore, one of these interactors named apolipoprotein A1 was found down regulated in serum of CTCL patients. Also, the identification of RBP-4 and RXR-β as TTR interactors might build a connection between the common tumour marker TTR and cell proliferation.

The present work shows the successful application of different methods for biomarker discovery in serum, cells and tissue. These potential markers were further characterised and after clinical validation might lead to new expertise in tumour biology and will serve as starting points in cancer treatment.

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Zhou, W., Ross, MM., Tessitore, A. et al. (2009). An initial characterization of the serum phosphoproteome. *J. Proteome Res.* **8**: 5523-5531.

7 Eidesstattliche Erklärung

Hiermit bestätige ich, Niko Escher,

dass mir die geltende Promotionsordnung der Fakultät bekannt ist;

dass ich die Dissertation selbst angefertigt und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben habe;

dass die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und das Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen;

dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe;

und dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Bei meiner Promotion haben mich bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts folgende Personen unterstützt: PD Dr. Christian Melle und Prof. Dr. Ferdinand von Eggeling

Jena, den 16.03.2010 _____

(Niko Escher)

8 Lebenslauf

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09.1991 - 08.1997	Friedrich-Fröbel-Gymnasium in Bad Blankenburg mit Abschluss Abitur

Grundwehrdienst

09.1997 - 06.1998	Bundeswehr in Ellwangen, Tätigkeit als Militärkraftfahrer auch im Bereich der Gefahrguttransporte und Organisation der Nachschubversorgung

Studium

09.1998 - 12.2004	Diplom Biologie Studium an der Friedrich-Schiller-Universität in Jena Hauptfach: allgemeine Zoologie Nebenfächer: Anthropologie und Genetik
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Wissenschaftlicher Mitarbeiter

01.2005 - 10.2007	Projekt mit dem Thema: Identifizierung und Evaluierung der mit ProteinChip array Technologie ermittelten tumorrelevanten Markern beim kutanen T-Zell-Lymphom (CTCL) und Nierenzellkarzinom
11.2007 - 07.2009	IZKF Verbundprojekt: Detektion und Identifizierung von Tumormarkern im Stroma und deren Relevanz bei der Metastasierung
09.2009 - 10.2009	Elternzeit
11.2009 - 01.2010	Weiterführung des IZKF Projektes und Arbeit an der Dissertation mit dem Titel „Detektion, Identifizierung und Charakterisierung tumorrelevanter Proteine in Serum, Gewebe und Zellen“
seit 03.2010	angestellt als Biologe in der Firma CLONDIAG, Jena

Weiterbildungen/Workshops

u. a.

06.2004	Protein Purification und ID in Guildford, UK
09.2004	Proteinreinigung im Labormaßstab
06.2005	Schreibwerkstatt-kreativ zum professionellen Text
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11.2006	Rhetorik – überzeugend Auftreten und Reden

Sprachen

Englisch sehr gut, Grundkenntnisse in Russisch und Spanisch

EDV

sehr gute Microsoft Office Kenntnisse, *Peptide Mass Fingerprint* Identifizierungssoftware wie Mascot oder Profound, Grundkenntnisse in SPSS

9 Publikationsliste / Poster / Vorträge

9.1 Publikationen

Detection and identification of protein interactions of S100 proteins by ProteinChip technology.
Lehmann R, Melle C, **Escher N**, von Eggeling F.
J Proteome Res. 2005 Sep-Oct;4(5):1717-21.

Identification of HNP3 as a tumour marker in CD4+ and CD4- lymphocytes of patients with cutaneous T-cell lymphoma.
Escher N, Spies-Weisshart B, Kaatz M, Melle C, Bleul A, Driesch D, Wollina U, von Eggeling F.
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Protein profiling of microdissected pancreas carcinoma and identification of HSP27 as a potential serum marker.
Melle C, Ernst G, **Escher N**, Hartmann D, Schimmel B, Bleul A, Thieme H, Kaufmann R, Felix K, Friess HM, Settmacher U, Hommann M, Richter KK, Daffner W, Täubig H, Manger T, Claussen U, von Eggeling F.
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Various members of the E2F transcription factor family interact in vivo with the corepressor alien.
Escher N, Kob R, Tenbaum SP, Eisold M, Baniahmad A, von Eggeling F, Melle C.
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Posttranslational modifications of transthyretin are serum markers in patients with mycosis fungoides.
Escher N, Kaatz M, Melle C, Hipler C, Ziemer M, Driesch D, Wollina U, von Eggeling F.
Neoplasia. 2007 Mar;9(3):254-9.

Detection and identification of transcription factors as interaction partners of alien in vivo.
Kob R, Baniahmad A, **Escher N**, von Eggeling F, Melle C.
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S100A8 Cellular Distribution in Normal Epithelium, Hyperplasia, Dysplasia and Squamous Cell Carcinoma and its Concentration in Serum
Oliver Driemel*, **Niko Escher***, Günther Ernst, Christian Melle, Ferdinand von Eggeling
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* beide Autoren zu gleichen Teilen an der Arbeit beteiligt

SELDI-TOF-MS meets BN-PAGE: Serum Biomarker discovery in Cutaneous T-cell Lymphoma (CTCL)
Niko Escher, Robert Kob, Martin Kaatz, Christian Melle, Christina Hipler, Mirjana Ziemer, Dominik Driesch, Uwe Wollina, Ferdinand von Eggeling
Einreichung bei Proteome Science in Vorbereitung

9.2 Poster

Detection of Protein-Protein-Interactions by ProteinChip Technology (SELDI)
Niko Escher, Christian Melle, Ferdinand von Eggeling;
2005 HUPO München

Detection of Protein-Protein Interactions of the Corepressor Alien; ***winner best poster award*** Robert Kob, **Niko Escher**, Juliane Kelm, Aria Baniahmad, Ferdinand von Eggeling and Christian Melle
2007 Proteomics Europe, Amsterdam

Post-tranlational modifications of TTT are serum marker in serum of patients with mycosis fungoides
Escher N, Melle C, von Eggeling F
2008 ESDR Zürich

Detection of Protein-Protein Interactions of the Corepressor Alien
Robert Kob, **Niko Escher**, Juliane Kelm, Aria Baniahmad, Ferdinand von Eggeling and Christian Melle
2008 HUPO Amsterdam

SELDI-TOF-MS meets BN-PAGE: Combining protein interaction studies and biomarker discovery
Niko Escher, Robert Kob, Christian Melle, Ferdinand von Eggeling,
2008 Amsterdam HUPO

Isolation and characterisation of tumour associated fibroblasts in urinary bladder carcinoma Astrid Enkelmann, **N.Escher**, M.Dürst, M.Knauer, J.Schubert, K.Junker
2008 ESUR Barcelona ; 18th Meeting of the European Society for Urological Research

Definition komplexer molekularer Muster der tumorassoziierten Fibroblasten metastasierender Tumoren
PD Dr. F. von Eggeling, **N. Escher**, G. Ernst, B. Schimmel, L. Wehder
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Changes in proteomic and genomic expression patterns in tumour associated fibroblasts by interaction with urinary bladder carcinoma cells
Enkelmann A., Heinzelmann J., Walter M., **Escher N**., Weidig M., Wunderlich H., Junker K.
2009 CNIO Madrid ; Bladder cancer research: The potential for a multidisciplinary integrative approach

Preparation and characterisation of tumour associated fibroblasts in urinary bladder carcinoma
Astrid Enkelmann, J.Sanjmyatav, M.Walter, **N.Escher**, M.Dürst, J.Schubert, K.Junker
2009 AEK Berlin ; 15th International AEK Cancer Congress

9.3 Vorträge

Mass Spectrometry Based Proteomic Profiling of Cutaneous T-Cell Lymphoma (CTCL)
2007, Dermatologie in Zürich

SELDI-TOF-MS meets BN-PAGE: Biomarker discovery in Cutaneous T-Cell Lymphoma (CTCL)
2007 Central European SELDI meeting in Berlin

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